Signaling pathways underlying zebrafish habenular development and connectivity

By
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Abstract

Recent studies have focused on the habenulae (Hb), bilateral nuclei in the epithalamus of the vertebrate forebrain due to their involvement in addiction and psychiatric disorders. The Hb consist of dorsal (dHb) and ventral (vHb) subnuclei, which are molecularly distinct and innervate different midbrain and hindbrain targets to modulate diverse behaviors. The specific neuronal populations that comprise the dHb, how these neurons arise and form appropriate connections with the principal midbrain target, the interpeduncular nucleus, are only partly understood.

My entry point to the study of habenular development was the characterization of a zebrafish mutation in the *wntless* (*wls*) gene, which encodes a chaperone protein necessary for secretion of Wnt morphogens. Through analyses of the habenular phenotype of *wls* homozygous mutants, I uncovered an unappreciated role for Wnt signaling in the generation of dHb progenitors. Previous studies on zebrafish mutants had shown that Wnt signaling is necessary to establish left-right (L-R) asymmetry of the dHb and for formation of the vHb. Although they develop small habenulae, L-R asymmetry is preserved in *wls* mutants, revealing temporally distinct requirements for Wnt activity. Hedgehog (Hh) and fibroblast growth factor (Fgf) signaling pathways are also involved in specification of dHb progenitors. Epistasis experiments indicate that Hh signaling is upstream of or parallel to Wnt signaling, which, in turn, defines the domain of Fgf signaling in the habenular region.

Previously, habenular progenitors were distinguished by expression of the *developing brain homeobox 1b* (*dbx1b*) and *chemokine (C-X-C motif), receptor 4b* (*cxcr4b*) genes. However, live-imaging, lineage tracing and long-term observation

indicate that *dbx1b* is transcribed by dHb progenitors, whereas *cxcr4b* transcripts localize to neural precursors located between the progenitors and mature neurons.

The function of Cxcr4b/chemokine signaling in dHb development was unknown. I determined that components of this pathway are expressed in cells surrounding the developing dHb and that Fgf signaling delimits their spatial domains. Finally, analyses of zebrafish mutants reveal that chemokine signaling directs posterior outgrowth of axons from dHb neurons.

Overall, my results provide new insights into the genetic network underlying dHb development and implicate chemokine signaling in the regulation of habenular axonal outgrowth.

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Dedication

To all those who have loved and supported me during this work.

Parents Susan and Robert Roberson,

Boyfriend Blake Caldwell

Best friend and sister Jessica Mann

'Children' Scoo Scoo, Miss Madison and Theo

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Abbreviations

BAC- Bacterial artificial chromosome

°C- degree Celsuis

CFP- cyan fluorescent protein

d- dorsal

DNA - deoxyribonucleic acid

dpf- days post fertilization

g – gram

GFP- green fluorescent protein

Hb- habenulae/ habenular nuclei

hpf- hours post fertilization

IPN – Interpeduncular nucleus

kb- kilobase pair

l- liter

µg – microgram

µl - microliter

µm- micrometer

nm- nanometer

PCR- polymerase chain reaction

%- percentage

RNA- ribonucleic acid

v- ventral

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Chapter 1: Introduction

Overview

The habenular nuclei, or habenulae (Hb), are conserved, bilateral structures in the dorsal diencephalon. They modulate important behaviors such as fear response, sleep and mating (Hikosaka, 2010; Aizawa et al., 2013; Huan et al., 1992; Sutherland, 1982; Modianos, 1974; Rodgers, 1976), and have been implicated in clinically relevant diseases such as Major Depressive disorder and Bipolar disorder and drug abuse, and in states such as anxiety/fear (Savitz et al., 2011; Lawson et al., 2016; Ranft et al., 2010; Klemm et al., 2004). Historically our understanding of habenular function has largely come from gross lesioning experiments (Modianos, 1974; Rodgers, 1976; Murphy et al., 1996; Murray et al., 1994), which can be imprecise and may damage other brain regions. Some information is known about the identity of habenular neurons and tracing experiments have revealed pre- and post-synaptic connections (Sutherland, 1982). However, the field has only recently begun to appreciate the molecular complexity of the Hb (deCarvalho et al., 2014; Quina et al., 2009;), which has reinvigorated investigations into their neural composition and the circuits in which they are involved.

In the past twenty years, interest in the Hb has been renewed in part due to their involvement in nicotine addiction and withdrawal, and their use as a model for studying left-right (L-R) asymmetry of the brain. The dorsal habenulae (dHb) of many non-mammalian vertebrates show prominent L-R differences in their size, gene expression, and neuronal connections.

Recent studies have also addressed the early specification and differentiation of the habenular nuclei. The genetic network regulating habenular development is complex,

often with a single signaling pathway involved in multiple, temporally distinct events. While some headway has been made, there is still significant work to be done to obtain a full picture of how the habenular nuclei form and the interactions between signaling pathways underlying their development. Some of the outstanding questions include how the habenulae generate the appropriate number of neurons, how neuronal diversity is achieved and how precise connectivity is established. A deeper molecular understanding of habenular development will not only provide fundamental information about diversification of brain nuclei, but will also enable the generation of precise tools to manipulate specific neuronal populations.

The zebrafish provides an excellent model to undertake such studies. Embryonic and larval zebrafish are transparent, which allows for the visualization of developmental processes in live animals. Additionally, zebrafish are amenable to genomic modification. Genetic constructs, such as those generated through recombination with bacterial artificial chromosomes (BACs) or by traditional cloning, can be introduced into the genome with relative ease using Tol2 transposition (Kawakami, 2005). The new generation of cutting-edge techniques, CRISPRs and TALENS, are also readily employed in the zebrafish (Hwang et al., 2013; Huang et al., 2011; Zhang et al., 2016; Ablain et al., 2015). These techniques can be used to generate *in vivo* reporters of gene expression or cell signaling events (Moro et al., 2013; Molina et al., 2007). In addition, transgenic tools can be used to perform lineage tracing, drive ectopic gene expression, destroy specific cell populations and even modulate neuronal activity (Felker et al., 2016; Subedi et al., 2014; Portugues et al., 2013; Horstick et al., 2016).

In the following sections, I outline our current state of knowledge regarding the specification of the habenular region in the dorsal forebrain and the molecular pathways that have been implicated in the development, left-right asymmetry and neural connections of the zebrafish habenulae. Where appropriate, I discuss these findings in the context of studies on the mammalian brain.

Specification and regionalization of the diencephalon

The habenular nuclei, along with the pineal gland, are part of the epithalamus, which arises from the anterio-dorsal region of prosomere 2 (p2) in the roof of the developing diencephalon (Hauptmann et al., 2002). Consequently, formation of the habenular nuclei depends on proper specification and regionalization of the diencephalon and its prosomeres. This is accomplished by input from signaling pathways along the dorso-ventral (D/V) and anterio-posterior (A/P) axes; the former by opposing gradients of bone morphogenic proteins (BMPs) and Sonic Hedgehog (Shh) signals and the latter by dynamic signaling centers that refine A/P subregions over time (Scholpp & Lumsden 2010; Hagemann & Scholpp, 2012; Wilson & Houart, 2004; Cavodeassi & Houart, 2012).

Wingless-INT proteins (Wnts) and Fibroblast growth factors (Fgfs) direct A-P patterning during the early subregionalization of the neural tube and establishment of the diencephalon. Canonical Wnt signals have been shown to promote posterior brain fates, such as the diencephalon, while simultaneously acting to antagonize forebrain fates (van de Water et al., 2001; Heisenberg et al., 2001). Non-canonical Wnts and inhibitors of canonical Wnt signaling are necessary for formation of anterior brain structures and to

oppose diencephalic fates (Heisenberg et al., 2001; Cavodeassi et al., 2005; Cavodeassi & Houart, 2012). Additionally, Fgf signals play an important role in the patterning of the forebrain (Shanmugalingam et al., 2000; Walshe & Mason, 2003; Cavodeassi & Houart, 2012). The opposing action of Fgf and Wnt signaling also determines the boundary between the telencephalon and diencephalon (Wilson & Houart, 2004; Houart et al., 2002).

The diencephalon is then divided into three distinct regions referred to as prosomeres (p1-p3), which themselves can be further subdivided into presumptive brain regions: p3 consists of the pre-thalamus (pTh), p2 of the epithalamus (comprised of the habenulae and pineal gland), zona limitas intrathalamica (ZLI) and thalamus, and p1 of the pre-tectum (PT) (Hauptmann et al., 2002; Puelles, & Rubenstein, 1993; Wullimann & Puelles, 1999). Boundaries between prosomeres are determined by gene expression and there is little mixing of cells between the prosomeres and the domains within them (Chatterjee et al., 2014, Staudt & Houart, 2007). A number of signaling centers are present along the A/P axis of the developing brain that control diencephalic regionalization (Fig. 1). Those most relevant for p2, and therefore epithalamic specification/habenular development, are the anterior neural boundary (ANB) in the very early telencephalon and later-arising mid-diencephalic organizer (MDO), located at the ZLI. The MDO is a source of Wnt, Fgf and Shh morphogens and plays an important role in creating the p2-p3 border and in patterning p2 (Chatterjee et al., 2014, Scholpp & Lumsden 2010; Hagemann & Scholpp, 2012; Wilson & Houart, 2004; Cavodeassi & Houart, 2012).

Proper positioning, size and molecular composition of the MDO is critical for thalamic and epithalamic development. It is an important source of Shh signals, which are thought to act antagonistically to specify epithalamic fates, while positively regulating rostral thalamic fates (Chatterjee et al., 2014). However, as described in the next section, an alternative model has been proposed for Shh action in epithalamic patterning (Halluin et al., 2016).

Habenular development

The habenulae (Hb) are highly conserved, bilateral brain structures that flank the pineal complex. They are comprised of two distinct nuclei, the dorsal (dHb) and ventral (vHb) nuclei that, respectively, are homologous to the medial and lateral habenular nuclei (mHb, lHb) of mammals (Aizawa et al., 2011). At larval stages, the habenular nuclei of zebrafish are found in a medial and lateral arrangement. Movements associated with eversion of the presumptive forebrain cause the lateral nuclei to rotate below the medial ones, creating the dorsal and ventral configuration seen in the adult brain (Aizawa et al., 2011).

Although the dHb and vHb are considered discrete structures, the question of whether they share a common developmental history has only recently been addressed. Work in zebrafish supports a model in which the vHb and dHb arise from spatiotemporally distinct progenitor populations (Beretta et al. 2013). A group of cells, referred to as the thalamic-epithalamic early projecting cluster (ThEPC), was identified posterior and lateral to the developing dHb (Beretta et al., 2013). Live imaging in conjunction with photoconversion of the ThEPC population revealed that it migrates

anteriorly to contribute to the vHb. Ablation of the ThEPC results in a smaller Hb, as determined by a transgenic line that labels both the vHb and dHb (Beretta et al., 2013). Specific loss of the vHb was not confirmed. The ability of ThEPC cells to migrate and contribute to the vHb is dependent on Wnt signaling. The vHb fail to form in zebrafish larvae homozygous mutant for the gene encoding Transcription Factor 7 Like 2 (Tcf7l2), a downstream effector of canonical Wnt signaling (Beretta et al., 2013). This finding reveals a key role for Wnt signaling in the development of the vHb; however, further study is needed to determine whether Wnt signals have a direct impact on ThEPC progenitor cells. In contrast to the vHb of zebrafish, little is known about the origin of the homologous lHb of mammals other than the timing of neurogenesis, which occurs between E12-15 in rats (Altman & Bayer, 1979).

Insights into the development of the dHb came from the molecular characterization of the thalamus in the mouse (Vue et al., 2007). Several of the genes described were also expressed in the presumptive epithalamus. One gene, *dbx1*, which encodes the *developing brain homeobox* transcription factor, was expressed at high levels specifically in the ventricular zone of the habenulae at E11.5 (Vue et al., 2007). Dbx1 protein is distributed in an anteriodorsal to posterioventral high to low gradient in the diencephalon, with high levels in the habenular ventricular zone and low levels at the ZLI (Vue et al., 2007). Short-term lineage tracing of $dbx1^{+}$ cells demonstrates that they give rise to a number of structures, including neurons in the habenular region (Vue et al., 2007). More recently, a zebrafish homolog of *dbx1* was identified as a marker of dorsal habenular progenitors (Dean et al., 2014). Expression of *dbx1b* is detected in the region of the developing habenulae and lineage tracing reveals that *dbx1b*-expressing cells give

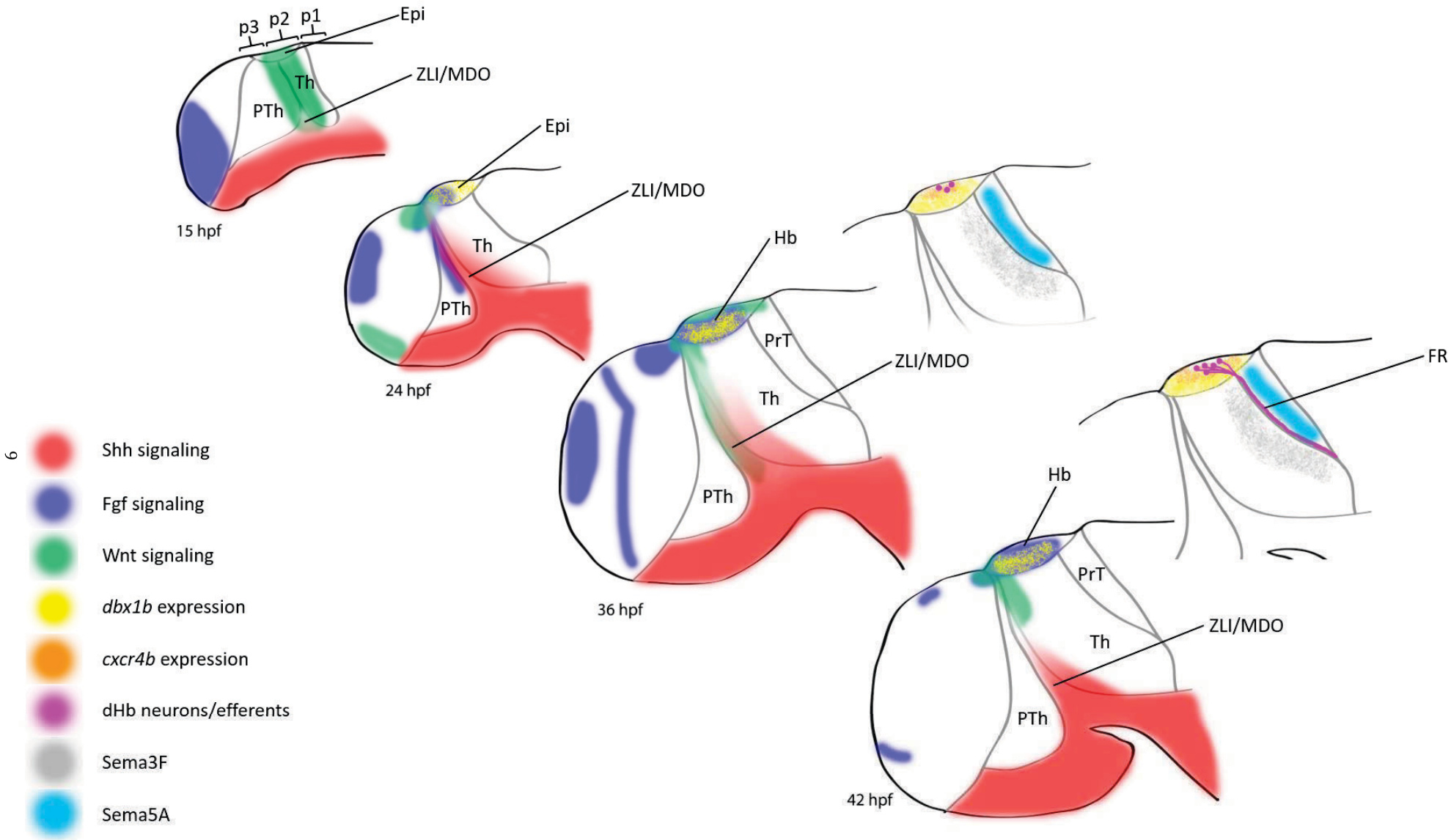
rise to neurons throughout the larval dHb (Dean et al., 2014; Fig. 1).

Prior to the description of *dbx1b* in habenular progenitors, *C-X-C chemokine receptor type 4b* (*cxcr4b*) was thought to be the earliest gene expressed in cells of the zebrafish developing Hb (Roussigné et al., 2009). Expression of *cxcr4b* is first detected at 28 hpf, in a similar pattern to more globally expressed pro-neural genes such as *neurogenin-1* (*neurog-1*) and *achaete-scute complex-like 1b*, but does not overlap with expression of known markers of differentiated post-mitotic neurons (Roussigné et al., 2009). Thus, the *cxcr4b*$^{+}$ population was proposed to correspond to either neural progenitors or newly born habenular neurons (Halluin et al., 2016; Roussigné et al., 2009). Comparisons between the *dbx1b* and *cxcr4b* expression patterns reveal that *cxcr4b* transcripts are present in only a subset of *dbx1b*-expressing cells at 36 hpf, as well as in cells more dorsolaterally positioned (Dean et al., 2014; Fig. 1). However, the relationship between the *cxcr4b*- and the *dbx1b*-expressing cell populations is not fully understood.

The ability to identify progenitors enabled earlier phenotypes to be detected in mutants with known defects in Hb morphology. In 2009, two groups reported that mutations in *fgf8* homologs result in a dramatic decrease in Hb size in the mouse and zebrafish (Martinez-Ferre & Martinez, 2009; Regan et al., 2009). The mouse mutant has significant abnormalities in brain morphology that complicate the interpretation of the Hb phenotype (Martinez-Ferre & Martinez, 2009), whereas the overall morphology and patterning of the brain are maintained in zebrafish *fgf8* mutants (Shanmugalingam et al., 2000; Regan et al., 2009; Dean et al., 2014). However, the zebrafish mutants show a reduction in *dbx1b* expression in the region of the developing dHb (Dean et al., 2014). A transgenic line activated by Fgf signaling confirmed that *dbx1b*$^{+}$ progenitors are

Fig. 1: Overview of diencephalic and habenular development:

Development of the zebrafish diencephalon from 15 hpf – 42 hpf. At 15 hpf Wnt (green), Fgf (blue), and Shh (red) signaling work to pattern the forebrain. Expression of and signaling from Shh has not yet expanded dorsally at the presumptive ZLI. By 24 hpf, Wnt, Fgf and Shh all influence the presumptive epithalamus, and expression of *dbx1b* (yellow) can be detected in the developing Hb. At 36 hpf, the developing Hb is likely influenced by Wnt, Fgf and Shh signaling. Expression of *cxcr4b* (orange; inset) has initiated in the Hb. The first dHb neurons appear (pink). Expression of *sema3f* (gray) and *sema5a* (blue) are found in at the border of p1 and p2 (Th and PrT). Expression of *dbx1b* and *cxcr4b* are still found in the developing Hb at 42 hpf. The number of dHb neurons has increased, and their efferents have begun to project towards their midbrain target. Neurons of the vHb have not yet been formed. Parapineal is not shown. Abbreviations: prosomere (p), epithalamus (Epi), prethalamus (pTh), thalamus (Th), pre-tectum (PrT), zona limitans intrathalamica (ZLI), mid-diencephalic organizer (MDO), habenula (Hb), fasciculus retroflexus (FR). Modified from Hageman & Scholpp, 2012.


p3
p2
p1
Epi
ZLI/MDO
Th
PTh
15 hpf
Epi
ZLI/MDO
Th
PTh
24 hpf
Hb
PrT
ZLI/MDO
Th
PTh
36 hpf
FR
Hb
PrT
ZLI/MDO
Th
PTh
42 hpf
Shh signaling
Fgf signaling
Wnt signaling
dbx1b expression
cxcr4b expression
dHb neurons/efferents
Sema3F
Sema5A

responsive to Fgfs (Dean et al., 2014). Furthermore, Fgf signaling is necessary to sustain expression of *dbx1b* in the developing dHb (Dean et al., 2014). Fgf signaling is thought to suppress dHb neurogenesis through control of the cyclin-dependent kinase inhibitor, *kip2,* and pro-progenitor factor *her6*, however this hypothesis remains to be tested directly (Dean, 2014).

Activation of a Fgf reporter is reduced in zebrafish mutant for the *mediator subunit 12* (*med12*) gene, whose product plays a fundamental role in connecting RNA Polymerase II (Pol II) to basal transcription factors so that Pol II is "poised" at particular genes to facilitate rapid induction of transcription (Wu et al., 2014; Yin & Wang, 2014). Zebrafish *med12* mutants have no discernable habenulae at 4 dpf and *dbx1b* and *cxcr4b* transcripts are absent in the region of the developing dHb at earlier stages (eg., 24, 36 hpf, respectively). Expression of *dbx1b,* however, recovers slightly by 48 hpf (Wu et al., 2014). It was proposed that loss of Med12 results in a delay in transcription of key genes and therefore a "missed window" for Hb specification (Wu et al., 2014). Med12 has also been implicated in regulating Wnt signaling (Rocha et al., 2010), which raises the interesting question of whether Med12 integrates Wnt and Fgf signaling in Hb development.

Shh signaling and the transcription factor Paired Box 6 (Pax6) genetically interact to influence formation of the Hb (Chatterjee et al., 2014; Halluin et al., 2016). Loss of Pax6 in mice or Pax6a in zebrafish reduces the size of *dbx1b* or *cxcr4b* expression domain, respectively (Chatterjee et al., 2014; Halluin et al., 2016). Additionally, overexpression of *pax6* in zebrafish results in the expansion of *cxcr4b* expression in the habenular region (Chatterjee et al., 2014). However, these studies differ on their

conclusions regarding the roles of and relationship between Shh signaling and Pax6 in Hb development.

Chatterjee et al. (2014) found that Shh represses Hb fates and that Shh signaling is downstream of *pax6.* Pax6 restricts *shh* expression at the ZLI. Accordingly, reduction of *pax6* transcripts results in an expansion of *shh* expression. This, in turn, leads to reduced *dbx1* and *cxcr4b* expression in the developing habenular region of mouse and zebrafish, respectively (Chatterjee et al., 2014). In zebrafish, loss of the Shh signaling component Smoothened (Smo) expands the habenular domain in zebrafish and a hypomorphic allele of *shh* in mice also produces an expanded domain of *dbx1* expression and larger habenulae (Chatterjee et al., 2014). However, the pineal gland is also altered in zebrafish *smo* mutants, suggesting that Shh signaling influences the development of the entire epithalamus and not just the habenulae (Chatterjee et al., 2014).

In contrast, recent work from Halluin et al. (2016) demonstrates that Shh signaling through Smo is required for the formation of the dHb. Zebrafish larvae homozygous for a mutation in *smo* fail to express *cxcr4b* or the *POU domain, class 4, transcription factor 1* (*pou4f1*) gene (also known as the *brain-specific homeobox/POU domain protein 3A, brn3a*), both markers of the Hb. Additionally, the authors claim that expression of *pax6* is downstream rather than upstream of Shh signaling, and that Pax6 function is required for habenular neurogenesis (Halluin et al., 2016).

The discrepancy between these studies is likely due to the use of different *smo* alleles, the expression analyses being performed at different stages and/or the imprecise designation of the habenular region. Further research is required to elucidate the precise

relationship between Pax6 and Shh in specification of the Hb region.

While most studies have focused on the role of morphogens and their downstream effects, it is worth noting that cell signaling outside of conventional morphogenetic pathways can also affect Hb development. One such example is the role of light and melatonin (de Borsetti et al., 2011). The Hb express opsins and melatonin receptors and they receive projections from light-sensing neurons (Bertolucci & Foa, 2004; Korf et al., 1986; Weaver et al., 1989). Light-responsiveness may influence Hb neurogenesis (de Borsetti et al., 2011). Zebrafish embryos raised in the dark have an increase in the number of *cxcr4b* expressing cells, but normal numbers can be restored by treatment with melatonin (de Borsetti et al., 2011).

The mature Hb contain a complex and diverse number of neural subtypes and discrete subdomains (deCarvalho et al., 2014; Quina et al., 2009). Despite our growing comprehension of how they form, it is still unclear how a seemingly homogeneous progenitor population acquires specific neural identity. One clue comes from a mutation in *pou4f1* (Quina et al., 2009), a gene expressed in almost all post-mitotic dHb neurons in mouse and zebrafish (Aizawa et al., 2007; Roussigné et al., 2009; deCarvalho et al., 2014; Quina et al., 2009). In *pou4f1* mouse mutants, expression of neurotransmitter receptors is altered; there is an increase in expression of GABA and glycine receptor subunits and a decrease in expression of serotonin, cholinergic, and somatostatinergic receptors (Quina et al., 2009). This mutant only touches the surface of what is likely to be a complicated network of genetic regulatory pathways that produces the diverse neuronal cell types of the Hb.

Left-right asymmetry of the dorsal habenulae

The prominent left-right (L-R) asymmetry of the dHb in the brains of zebrafish, and of other teleosts and non-mammalian species (Braitenberg, 1970; Concha & Wilson, 2001), adds another level of complexity to their neuronal composition. In the zebrafish, expression of the Nodal-related gene *southpaw* (*spaw*) in the left lateral plate mesoderm (LPM) at the 16 somite stage has an impact on the later asymmetry of the viscera and brain (Long et al., 2003; Rebagliati et al., 1998). Asymmetric Nodal signaling in the LPM activates transcription of several Nodal pathway genes in the left diencephalon by 20 hpf (Long et al., 2003), including *nodal related 2* (*ndr2*), *lefty1* (*lft1*) which encodes a Nodal antagonist, and *paired-like homeodomain 2* (*pitx2*), which encodes a Nodal regulated transcription factor (Liang et al., 2000; Concha et al., 2000). This asymmetric expression was shown to localize to the left side of the pineal anlage (Liang et al., 2000).

At approximately 36 hpf, a string of cells migrates leftward from the pineal anlage to form the presumptive parapineal organ (Concha et al., 2003; Snelson et al., 2008). The mature neurons of the parapineal selectively innervate the left dHb (Concha et al., 2000; Concha et al., 2003). The left and right dHb differ with respect to their size, organization, gene expression and patterns of efferent and afferent innervation (Halpern et al., 2003; Aizawa et al., 2005; Aizawa et al., 2007; Roussigné et al., 2009; Miyasaka et al., 2009). The directionality of parapineal position and dHb asymmetry typically correspond. This suggests a causal relationship in which the parapineal directs the left dHb to develop different properties from the right dHb (Aizawa et al., 2011; Gamse et al., 2003; Concha et al., 2003). Indeed, laser ablation of the parapineal organ causes the left dHb to adopt a right dHb identity (i.e., right-isomerized; Gamse et al., 2003; Concha et al., 2003).

Disruption of parapineal development and/or migration of precursors also perturbs habenular laterality. Loss of the transcription factor T-box 2 (Tbx2) (Snelson et al., 2008) or the Mediator complex subunit 12 (Med12) (Wu et al., 2014) affects migration and parapineal cell fate or parapineal cells that fail to mature, respectively. Similar to asymmetry defects caused by parapineal ablation, defective parapineal development in *tbx2* mutants causes right isomerization of the dHb (Snelson et al., 2008).

A number of signaling pathways have been implicated in epithalamic asymmetry, such as Nodal, Wnt, Fgf, and Notch (Carl et al., 2007, Hüsken et al., 2014; Aizawa et al., 2011; Regan et al., 2009; Rebagliati et al., 1998; Gamse et al., 2003; Concha et al., 2000; Liang et al., 2000). In zebrafish, genes involved in the Nodal signaling pathway are not necessary for the establishment of L-R asymmetry, but rather for the directionality of this asymmetry. Loss of Nodal signaling results in L-R randomization of the position of the parapineal (Concha et al., 2003; Gamse et al., 2003) and base of the pineal stalk (Liang et al., 2000). Approximately half of larvae show a L-R reversal in habenular asymmetry (Concha et al., 2003; Gamse et al., 2003; Gamse et al., 2005; Facchin et al., 2009). For example, the *potassium channel tetramerisation domain containing 12.1* (*kctd12.1*) is predominantly expressed in the left dHb but depletion of Spaw with antisense morpholinos increases the frequency in which *kct12.1* is predominantly expressed on the right (Facchin et al., 2009). Interestingly, the function of the Nodal pathway appears to have diverged over evolution. In more ancestral species, such as the catshark and the lamprey, Nodal signaling is required for asymmetry of the epithalamus and not just its directionality (Lagadec et al., 2015).

Fgf8, in conjunction with Nodal, is also believed to regulate the L-R positioning

of the parapineal. In zebrafish *fgf8a* mutants, the parapineal fails to migrate leftward and the dHb are smaller and right isomerized (Regan et al., 2009). Fgf8-coated beads were sufficient to restore parapineal migration. Expression of *fgf8a* is reported to have a subtle asymmetry, with slightly higher levels in the right epithalamus, and this asymmetric expression is dependent on Nodal signaling. These findings indicate that Fgf signaling promotes the asymmetric migration of the parapineal precursors from the midline.

Loss of Axin1, a Wnt scaffolding protein required for the degradation of β-catenin, and the concurrent increase in Wnt signaling, produces bilateral expression of *lft1* and *pitx2*in the dorsal diencephalon. However, *spaw* expression in the LPM is unaffected (Carl et al. 2007). This finding indicates that Wnt signal transduction regulates expression of Nodal pathway components in the left diencephalon, but not Nodal signaling in the LPM. This bilateral activation of Nodal signaling randomizes the direction of parapineal migration. Unexpectedly, the dHb are right isomerized, regardless of parapineal position (Carl et al., 2007). This is likely due to a delay in parapineal migration observed in *axin1* mutants. Together, these data suggest that there is a small window in which the parapineal can influence habenular L-R asymmetry.

Pharmacological overactivation of the canonical Wnt pathway late in gastrulation (8.5 hpf), partially phenocopies the *axin1* mutant (Carl et al., 2007). Nodal gene expression is bilaterally activated, but L-R asymmetry of the dHb is unaffected. Conversely, over-activation of Wnt signaling at the 14-somite stage (16 hpf) fully phenocopies the *axin1* mutant, as both Nodal expression and dHb asymmetry are altered. This reveals two temporally distinct functions of Wnt signaling in regulating habenular asymmetry- an early role in regulating the expression of genes in the Nodal pathway in

the dorsal diencephalon, and a later role in regulating Nodal genes and habenular laterality. The latter may also involve Tcf7l2, the Wnt signaling effector involved in the development of the vHb (Hüsken et al., 2014). Zebrafish larvae with a mutation in *tcf7l2* have left-isomerized dHb, despite asymmetry of Nodal gene expression, parapineal migration, habenular precursor number and neurogenesis (Hüsken et al., 2014). Tcf7l2 protein is detected bilaterally within habenular neurons after 35 hpf. Thus, genetic analyses indicate that activation of Wnt signaling is required for right dHb fates, and an absence of Wnt signaling results in left dHb fates (Carl et al., 2007; Hüsken et al., 2014). Additionally, as described in this thesis, Wnt signaling has another unexpected role in the regulation of dHb precursors.

Asymmetric activation of the Notch pathway regulates neurogenesis, which underlies the formation of dHb L-R asymmetry (Aizawa et al., 2007; Aizawa et al., 2011). Notch signaling inhibits neurogenesis, and reduced signaling leads to excess neurogenesis. Therefore, the outcome of asymmetric activation of Notch signaling is asymmetric neurogenesis and differently sized neuronal sub-regions in the left and right dHb (Aizawa et al., 2007; Aizawa et al., 2011).

The dHb can be further divided into two subdomains, the medial dHb (dHbM) and lateral dHb (dHbL). The dHbL, distinguished by labeling from the transgenic line *Tg(pou4f1-hsp70l:GFP)* or by expression of *kctd12.1* (Aizawa et al., 2005; Aizawa et al., 2007), is larger in the left dHb than the right dHb. The dHbM, defined by labelling from *Tg(nptx2:Gal4-VP16)* or expression of *potassium channel tetramerisation domain containing 12.2* (*kctd12.2*), is larger in the right dHb than in the left dHb (Gamse et al., 2005; Agetsuma et al., 2010; Aizawa et al., 2007). Early-born neurons (labeled by BrdU

between 24-36 hpf) predominantly contribute to the dHbL, while later-born neurons (labeled by BrdU between 36-72 hpf) predominantly contribute to the dHbM (Aizawa et al., 2007). Experiments that delay neurogenesis suggest that the dHbM fate is repressed early in development and de-repressed at 36 hours, after which neurons of dHbM or dHbL fates are generated concurrently (Aizawa et al., 2007).

Habenular efferent projections: forming the fasciculus retroflexus

Early after their birth, habenular neurons extend their axons posteriorly. These habenular efferents join the fasciculus retroflexus (FR), a prominent nerve bundle that projects posteriorly and ventrally between prosomeres 1 and 2. The rodent FR is comprised of a core and a sheath; medial habenular efferents are found in the core while the sheath is comprised of lateral habenular efferents and afferents from the ventral tegmental area (Bianco & Wilson, 2009; Schmidt et al., 2014). The majority of dHb (or mammalian mHb) axons terminate at their major midbrain target, the unpaired interpeduncular nucleus (IPN). Neurons in the vHb (mammalian lHb) project further to the raphe nucleus (Aizawa et al., 2011; Sutherland, 1982).

In zebrafish, habenular axons can be detected as early as 2 dpf by immunolabeling against Kctd12.1 and Kctd12.2 (Kuan et al., 2007; Gamse et al., 2005). Some axon terminals have reached the IPN prior to 3 dpf (Fig. 1; Kuan et al., 2007; Gamse et al., 2005). These early projecting axons likely emanate from dHb neurons as the vHb begins to form at 2 dpf and is not fully formed until 4 dpf (Beretta et al., 2013). In the rat, the first habenular efferents are detected at E13 and they reach the caudal diencephalon by

E14 (Funato et al., 2000). In contrast to zebrafish, these early projections in the rat are efferents from lHb neurons, which form between E12-15. The medial habenular neurons do not form until E14-18 (Funato et al., 2000).

How habenular neurons connect with their midbrain and hindbrain targets is only partly understood. The family of semaphorins is well represented in the list of guidance molecules that are involved in directing habenular axons to fasciculate in the FR, and guiding them along their path to midbrain targets. Sema3f and Sema5a both play roles in the fasciculation of habenular efferents in the FR (Giger et al., 1998; Sahay et al., 2003; Funato et al., 2000; Kantor et al., 2004). Sema3f is expressed along the rostral border of prosomere 1 (Fig. 1, and it binds to the receptor Neuropilin-2 (Npn2), which is expressed in both the lateral and medial habenulae (Funato et al., 2000; Giger et al., 1998; Giger et al., 2000). Morpholinos against zebrafish *sema3f* did not result in any apparent defect in the FR (Kuan et al., 2007). However, in the rodent, the FR of *sema3f* mutants is defasciculated and wider, although axons still successfully project towards the IPN (Sahay et al., 2003; Chen et al., 2000). The FR of *npn2* mutants is smaller and consists of multiple smaller fascicles (Giger et al., 2000). Analyses of habenular axons in rats indicate that Sema3f normally acts as a chemorepellant that prevents Npn2-expressing habenular axons from entering prosomere 1 (Sahay et al., 2003; Chen et al., 2000; Giger et al., 2000; Funato et al., 2000).

Sema5a has been suggested to play a bifunctional role in axon pathfinding, depending on the extracellular context (Kantor et al., 2004). It acts in an inhibitory manner when presented with chondroitin sulfate proteoglycans (CSPGs). Conversely, Sema5a becomes an attractive cue in the presence of heparan sulfate proteoglycans

(HSPGs). HSPGs are found on the surface of extending FR axons, while CSPGs are found in the diencephalon. Expression of Sema5a is found in prosomere 2 by E13.5, and within the habenulae by E15. Loss of Sema5a results in aberrant axon projection into p2, and failure habenular axons to reach midbrain targets. Co-culture experiments of habenular and p2 explants (Funato et al., 2000) further support the hypothesis that p2 is non-permissive to Sema5a-expressing habenular axons (Fig. 1)
through contact-mediated interaction with CSPGs. Moreover, the attractive HSPGs found on habenular axons promote axon fasciculation in the FR (Kantor et al., 2004).

A third member of the Semaphorin family, Sema3d, and its receptor Neuropilin1a (Nrp1a), plays a role in innervation of the dorsal versus ventral IPN in zebrafish (Kuan et al., 2007). Nrp1 is not found in the habenulae of rats (Funato et al., 2000), but *nrp1a* is expressed in the left dHb of 2 dpf zebrafish, (Kuan et al., 2007). The dHbL, the larger of the two subdomains of the left dHb, innervates the dorsal IPN (dIPN), a characteristic that is not shared with the dHbM, the prominent subdomain of the right dHb (Gamse et al., 2005; Kuan et al., 2007). Sema3d is an attractive cue, expressed along the FR, and dorsal to the IPN (Kuan et al., 2007). Axons of left dHbL neurons expressing *nrp1a* bifurcate and extend processes dorsally to innervate the dIPN. Morpholino-mediated knockdown of either *sema3d* or *nrp1a* transcripts results in reduced innervation of the dIPN (Kuan et al., 2007).

Netrin and its receptor Deleted in Colorectal Carcinoma (DCC) are also involved in the guidance of habenular axons to the IPN (Funato et al., 2000; Schmidt et al., 2014). DCC is expressed in the FR of stage E14 rats (Funato et al., 2000) and in the medial habenulae of stage E16.5 mice (Schmidt et al., 2014; Quina et al., 2009). The netrin1

ligand is present in the ventral caudal diencephalon (VCD) (Funato et al., 2000) and in the lateral habenulae and their dopaminergic afferents (Schmidt et al., 2014; Quina et al., 2009). In the VCD, the role of Netrin1 is to attract habenular axons ventrally and caudally towards their midbrain targets (Funato et al., 2000). Habenular-derived Netrin1 is not believed to guide efferents but rather regulates afferent innervation of the lHb (Schmidt et al., 2014). Loss of either DCC or Netrin1 results in a reduction of mHb efferents in the FR (Schmidt et al., 2014), which instead project to the dorsal roof of the Hb (Schmidt et al., 2014).

While the function of Semaphorins and Netrin/DCC have been examined, there are many more axon guidance cues that may have important roles in directing the outgrowth of habenular axons, mediating fasciculation in the FR or the innervation of their targets. Candidates from mass spectrometry and immunolabeling experiments include the Roundabout guidance receptor 3 (Robo3) and Glypican2, which are found in axons of the medial habenulae, and the close homolog of L1 (CHL1) protein that present in the sheath of the FR, which is populated with axons of the lateral habenula (Schmidt et al., 2014). The Frizzled3/PCP pathway has also been implicated in the formation of the FR, as the axon bundles are smaller in Fz3 mutants compared to their WT siblings (Hua et al., 2014). In *pou4f1* mouse mutants, the trajectory of habenular axons is normal but they fail to innervate the IPN (Quina et al., 2009).

Although we are beginning to understand the mechanisms and signaling pathways underlying habenular efferent outgrowth, pathfinding and target innervation, many questions remain. What controls the initial posteriorly directed extension of habenular axons? How are the various molecular components of axon guidance regulated in relation

to signaling pathways such as Hh, Wnt and Fgf, which have been implicated in earlier aspects of habenular development? Do different neuronal cell types found in the habenulae have specific target regions along the IPN and raphe that require fine-tuning of axon guidance? Understanding such fundamental issues has important consequences for deciphering dHb-IPN connectivity and ultimately the diverse functions of their neuronal populations.

In the following chapters I will describe my work on the development of the dorsal habenular nuclei in zebrafish. Chapter 2 is a collaborative effort in which I present the isolation and characterization of a mutation in the zebrafish *wntless* gene and discuss the cellular basis for the reduction in size of the dHb in homozygous mutants. In Chapter 3, I describe the roles of multiple signaling pathways in the formation of habenular progenitors, precursors, and neurons, and how their interactions reveal a new function for a chemokine signaling pathway in directing the proper outgrowth of habenular axons. General conclusions from my work are provided in Chapter 4, as well as some of the outstanding questions, whose answers will contribute to a more complete understanding of habenular development.

Chapter 2: Distinct requirements for Wntless in habenular development

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ABSTRACT

Secreted Wnt proteins play pivotal roles in development, including regulation of cell proliferation, differentiation, progenitor maintenance and tissue patterning. The transmembrane protein Wntless (Wls) is necessary for secretion of most Wnts and essential for effective Wnt signaling. During a mutagenesis screen to identify genes important for development of the habenular nuclei in the dorsal forebrain, we isolated a mutation in the sole *wls* gene of zebrafish and confirmed its identity with a second, independent allele. Early embryonic development appears normal in homozygous *wls* mutants, but they later lack the ventral habenular nuclei, form smaller dorsal habenulae and otic vesicles, have truncated jaw and fin cartilages and lack swim bladders. Activation of a reporter for ß-catenin-dependent transcription is decreased in *wls* mutants, indicative of impaired signaling by the canonical Wnt pathway, and expression of Wnt-responsive genes is reduced in the dorsal diencephalon. Wnt signaling was previously implicated in patterning of the zebrafish brain and in the generation of left-right (L-R) differences between the bilaterally paired dorsal habenular nuclei. Outside of the epithalamic region, development of the brain is largely normal in *wls* mutants and, despite their reduced size, the dorsal habenulae retain L-R asymmetry. We find that homozygous *wls* mutants show a reduction in two cell populations that contribute to the presumptive dorsal habenulae. The results support distinct temporal requirements for Wls in habenular development and reveal a new role for Wnt signaling in the regulation of dorsal habenular progenitors.

INTRODUCTION

The habenulae are bilaterally paired nuclei in the epithalamus that connect the limbic forebrain to the mid- and hindbrain. In mammals, the habenulae consist of medial and lateral nuclei, which have been implicated in a wide variety of behaviors including anxiety, sleep and reward (Aizawa, 2013; Hikosaka, 2010; Viswanath et al., 2014) and in conditions such as addiction, bipolar disorder and depression (Ranft et al., 2010; Savitz et al., 2011; Viswanath et al., 2014). Despite their functional importance, little is known about the development of the habenular nuclei.

Recently, the zebrafish has emerged as a valuable model to study habenular development. Zebrafish possess dorsal (dHb) and ventral (vHb) habenular nuclei, equivalent to the medial and lateral habenula of mammals, respectively (Aizawa et al., 2011). A recent study demonstrated an essential role for Wnt signaling in formation of the ventral habenulae (Beretta et al., 2013). Wnt signaling is also thought to underlie the prominent left-right (L-R) differences in the organization, gene expression, and connectivity of the dorsal nuclei. A mutation in *axin1*, which encodes a negative regulator of Wnt/ ß-catenin signaling, causes both dorsal habenulae to adopt a right habenular identity as defined by gene expression and axonal targeting (Carl et al., 2007). Conversely, a mutation in *tcf7l2*, a Wnt-dependent transcriptional activator, results in the opposite phenotype, with both habenulae displaying features characteristic of the left nucleus (Hüsken et al., 2014). These findings suggest that the level of canonical Wnt signaling is a critical factor in the formation of habenular L-R differences. Although canonical Wnt signaling is essential for development of the ventral nuclei and for L-R

asymmetry of the dorsal nuclei, the precise Wnts that mediate these functions and their cells of origin are unknown.

For secretion and effective signaling, Wnt proteins require a lipid modification that renders them hydrophobic and insoluble (Ke et al., 2013; Takada et al., 2006). The chaperone protein required for Wnt secretion is Wntless (Wls) (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006), a transmembrane protein that hides the lipid modification in a lipocalin fold and escorts the Wnt protein through the secretory pathway to the cell membrane (Das et al., 2012; Willert & Nusse, 2012). At the cell membrane, the Wnt is released from Wls, which is then recycled back to the endoplasmic reticulum via the Golgi to participate in further rounds of Wnt trafficking (Franch-Marro et al., 2008; Yu et al., 2014). It is currently thought that Wls is required for secretion of almost all Wnts and, therefore, necessary for canonical and non-canonical Wnt signaling (Coudreuse & Korswagen, 2007; Najdi et al., 2012, Port & Basler, 2010). This is supported by the severe, early phenotypes of *wls* homozygous mutant mouse embryos and maternal zygotic *Drosphila* mutants (Banziger et al., 2006; Bartscherer et al., 2006; Carpenter et al., 2010; Fu et al., 2009; Goodman et al., 2006).

In the course of a mutagenesis screen to identify genes that control habenular development, we isolated a mutation in the zebrafish homolog of *wls*. In homozygous mutant embryos, the dorsal habenulae are reduced in size and the ventral habenulae are absent, whereas other regions of the brain appear unaffected. Surprisingly, the dorsal habenulae retain their L-R differences, suggesting that maternally provided Wls fulfills this requirement for Wnt signaling or that generation of habenular asymmetry is a Wls-independent process. However, early signaling is not sufficient to regulate habenular

precursor cell populations, which are reduced in number in *wls* mutants. Our findings suggest that there are three distinct roles for Wnt signaling in habenular development, an early requirement for L-R asymmetry and later functions in the specification of the ventral habenulae and in the regulation of dorsal habenular precursors.

Materials and Methods

Zebrafish strains and husbandry

Zebrafish were maintained at 28°C on a 14:10 light: dark cycle. The wildtype AB strain (Walker, 1999), the N-ethyl-N-nitrosourea (ENU) induced mutations *wls*^{c186} and *wls*^{fh252} and the transgenic lines *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} (Moro et al., 2012), *TgBAC(dbx1b:GFP)* (Kinkhabwala et al., 2011) and *TgBAC(gng8:Eco.NfsB- 2A-CAAX-GFP)* (deCarvalho et al. 2013) were used. Maintenance and care of zebrafish and experimental procedures were performed in accordance with the Carnegie Institutional Animal Care and Use Committee.

ENU mutagenesis and gene identification

Mutations were induced by exposure of AB males to ENU as previously described (Haffter 1996). Mutant phenotypes were identified in the F3 generation following screening for expression of *kctd12.1*. Carriers were outcrossed to AB fish to maintain and expand the line. To generate a mapping panel, c186 heterozygous males were mated to WIK (Rauch 1997) females. The position of the c186 lesion was determined by bulked segregation analysis using simple sequence repeat length polymorphisms (SSLP) (Talbot and Schier, 1999). Sequences for SSLP primers are provided at *http://www.zfin.org*, with the exception of the ys-Lg2-12 primers (5'- TCC AGC AGT CAA ATC AGG TG -3' and 5'- TCC AGC AGT CAA ATC AGG TG -3'), which were designed based on genomic DNA sequence. Exons of the zgc:64091 transcript (NCBI accession# NM_213146.1) were PCR amplified for DNA sequencing using the following primers:

exon 1: 5’-GAGCCTGGCCGTGTGACGTCA-3’ and

5’- ACCAAACTGGAAACACACTGTGCA-3’

exon 2: 5’- GAATCGAAAATGTAATCGAATAGAGG-3’ and

5’-AATCGCCAATATGGATGAGGAG-3’

exons 3 and 4: 5’- CAGTGCAGCAAC GTTTACTGTTT-3’ and

5’-TGTGCGTTTTAGACATGCATCC-3’

exon 5: 5’-AGCAAACAACGATACCCATCAA-3’ and

5’-AGAACACCCAGACAACCACAA- 3’

exon 6: 5’-TGTTTCTTGGTGAGGTGTGCT-3’ and

5’-ATTCTGCACCAATTGATCCAC-3’

exons 7 and 8: 5’-AAAAAAGTGGGTCCAACCTGGTAC-3’ and

5’-ACAGCACAGCAACCATCACAAT-3

exon 9: 5’-GACCATACTATTGTGATGGTTG-3’ and

5’-TGCCTTCTGCATCACTTTTGAC-3’.

exon 10 and 11: 5’-GTTTGCTCGTTACTGAACCCAT-3’ and

5’-CACACTATTTACTTATGCACTTAC CCA-3’.

The fh252 allele was generated by TILLING as described previously (Draper et al., 2004).

Genotyping

Genotyping of the c186 allele was performed using a derived cleaved amplified polymorphic sequence (dCAPS). Genomic DNA was PCR amplified using forward (5’-GACTGAGAGGAACCGCTTTTCAGTGTTCT-3’) and reverse (5’-AGAAAGGATTCT

TTAGCTGTACTCCTCTGC-3') primers. The forward primer contains a mismatch, generating a DNA fragment that is sensitive to XbaI digestion in the c186 allele but not in WT.

RNA injection

The zgc:64091 clone corresponding to full-length *wls* cDNA was purchased from the ATTC Resource Center, Manassas, VA. Sense RNA was produced using the mMESSAGE mMachine Kit (Ambion). For mutant rescue, approximately 1 nL of RNA (0.3 ng/nL in 0.2% phenol red and sterile water) was injected into zebrafish embryos at the 1-2 cell stage.

Bioinformatic analyses

Low homology screens to identify additional *wls* genes in the zebrafish genome were performed using BLAST and tBLASTx algorithms (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Syntenic regions were found by examining the genes flanking *wls* and searching for duplicated copies of their coding sequences in the zebrafish genome or for homologous genes in the genomes of other teleost species (www.ensembl.org).

RNA *in situ* hybridization and immunofluorescence

Techniques for colormetric and fluorescent in situ hybridizations were performed as described previously (deCarvalho et al., 2013). RNA probes for *pax6* (Krauss et al., 1991), *dlx2a* (Akimenko et al., 1994), *fgf8* (Fürthauer et al., 1997), *wls, neurog1, cxcr4b*

(Thisse et al., 2001), *olig2* (Park et al., 2002), *nkx2.2* (Thisse & Thisse, 2004), *dbx1b* (Gribble et al., 2007), *kctd12.1*, *kctd12.2*, *f-spondin*, (Gamse et al., 2003; Gamse et al., 2005), *nrp1a* (Yu et al., 2004), *vachtb* (Hong et al., 2013) and *ano2* (deCarvalho et al., 2014) were synthesized according to the published methods. cDNA fragments for *lef1* and axin2 were generated by RT-PCR using primers 5'-TGGCATGCTTTATCTCGGGAA-3' and 5'- GTCAAAGATGCCTATTTATTTCCA-3'and 5'-AAGTCGCACAGTTTGGAACC-3' and 5'-CACATCATCGGCTATTGGCT-3', respectively. The amplified fragments were cloned into pCRII by TOPO TA-cloning (Invitrogen K4600-01).

Immunolabeling to detect GFP or pH3 was performed as described previously (deCarvalho et al., 2013) using rabbit GFP antisera (Torrey Pines Biolabs) or anti-phospho-Histone H3 (ser10) antibody (Millipore), respectively, and Cy3-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch). To quantify the number of cells undergoing mitosis in the presumptive dorsal habenulae, ph3 immunolabeling was performed on WT and c186 mutant embryos carrying the *TgBAC(dbx1b:GFP)* transgene that labels this brain region. From a confocal z-stack, 3D images were processed using Imaris software (Bitplane) and the volume of the *dbx1b:GFP* habenular domain was calculated using the 'surfaces' function. Only $pH3^+$ nuclei found within this domain were counted using the 'spots' function, which was set to identify labeled spheres approximately 5 μm in diameter. The number of labeled nuclei was normalized to habenular volume and a two-tailed t-test was used to compare mutant and WT values.

Alcian Blue staining

Larvae were collected at 6 dpf, fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight and stored in PBS at 4°C. Alcian Blue (0.1% in 0.37% hydrochloric acid (HCl), 70% ethanol) was used to label cartilage according to standard methods (Schilling et al., 1996).

Microscopy

Bright field images were collected on a Zeiss Axioskop with an AxioCam HRc camera. Images incorporating both bright field and fluorescence were obtained using a Zeiss AxioZoom.V16 fitted with an AxioCam MRm. Confocal images were acquired with upright or inverted Leica SP5 microscopes using 40x oil immersion and 25x water immersion lens, respectively.

RESULTS

Recovery of mutations in the zebrafish *wntless* gene

The c186 mutation was discovered in a mutagenesis screen to identify genes involved in the development and/or L-R asymmetry of the zebrafish habenular nuclei, as determined by altered expression of the *potassium channel tetramerisation domain containing 12.1* gene (*kctd 12.1*, formerly known as *leftover*; Gamse et al., 2003). Homozygous mutants have a significantly reduced domain of *kctd12.1* expression compared to wild type (WT) siblings, indicative of smaller dorsal habenular nuclei. However, the L-R difference in the distribution of *kctd12.1* transcripts is preserved (Fig. 2A,E). During early embryonic development c186 mutants are morphologically indistinguishable from their WT siblings (data not shown), but by 4 days post fertilization (dpf) they exhibit notable defects such as smaller otic vesicles, pectoral fins and jaw cartilages (Fig. 2B-D,F-H). The mutants fail to develop swim bladders and die by approximately 12 dpf.

Recombination mapping of the c186 mutation placed it between z13620 (1 recombinant out of 1238 meioses) and z60815 (13/2066) on chromosome 2 (Fig. 2I), in the vicinity of the zebrafish homologue of the *Drosophila wntless* (*wls*) gene (Jin et al., 2010). Positional cloning followed by DNA sequencing verified that the mutation resulted from a single base change (G to A) in the seventh exon of zebrafish *wls*, leading to a premature stop codon. To verify that the exon 7 lesion was indeed responsible for the mutant phenotype, we generated a second allele, fh252, by Targeted Induction of Local Lesions In Genomes (TILLING; refer to Draper et al., 2004). The fh252 allele is a nonsense mutation in the third exon of the *wls* homolog, (Fig. 2I). Homozygous fh252

Fig. 2: Isolation of mutant zebrafish *wls* allele

(A-D) WT and (E-H) c186 homozygous mutants. (A, E) The c186 mutation was isolated through an RNA *in situ* hybridization screen using the asymmetrically expressed *kctd12.1* gene. Dorsal views, 4 dpf. (B, F) Bright field images at 6 dpf. (C, G) Alcian blue staining of jaw cartilages and (D, H) pectoral fins at 6 dpf. (I) Genetic map of the chromosome 2 region where the two ENU induced lesions are located. The c186 allele is flanked by microsatellite markers z13620 and z60815 (recombination frequencies indicated by numbers in parentheses). Three genes were annotated in this interval: *gadd45aa* (i); *gng12* (ii) and *wls/gpr177* (iii). Nonsense mutations in the fh252 (C to T) and c186 (G to A) alleles reside in exons 3 and 7, respectively. (J-M) Rescue of mutant phenotypes by injection of *wls* mRNA. Lateral views of WT (J), homozygous c186 mutant (K) and rescued c186 mutant (L) *TgBAC(gng8:Eco.NfsB- 2A-CAAX-GFP)* larvae at 7 dpf. The transgene labels habenular neurons and their axons with membrane-tagged GFP. (J'-L') are dorsal views of the dorsal habenular nuclei. (M) Genotyping of larvae was used to confirm mutant rescue (refer to Materials and Methods for details).

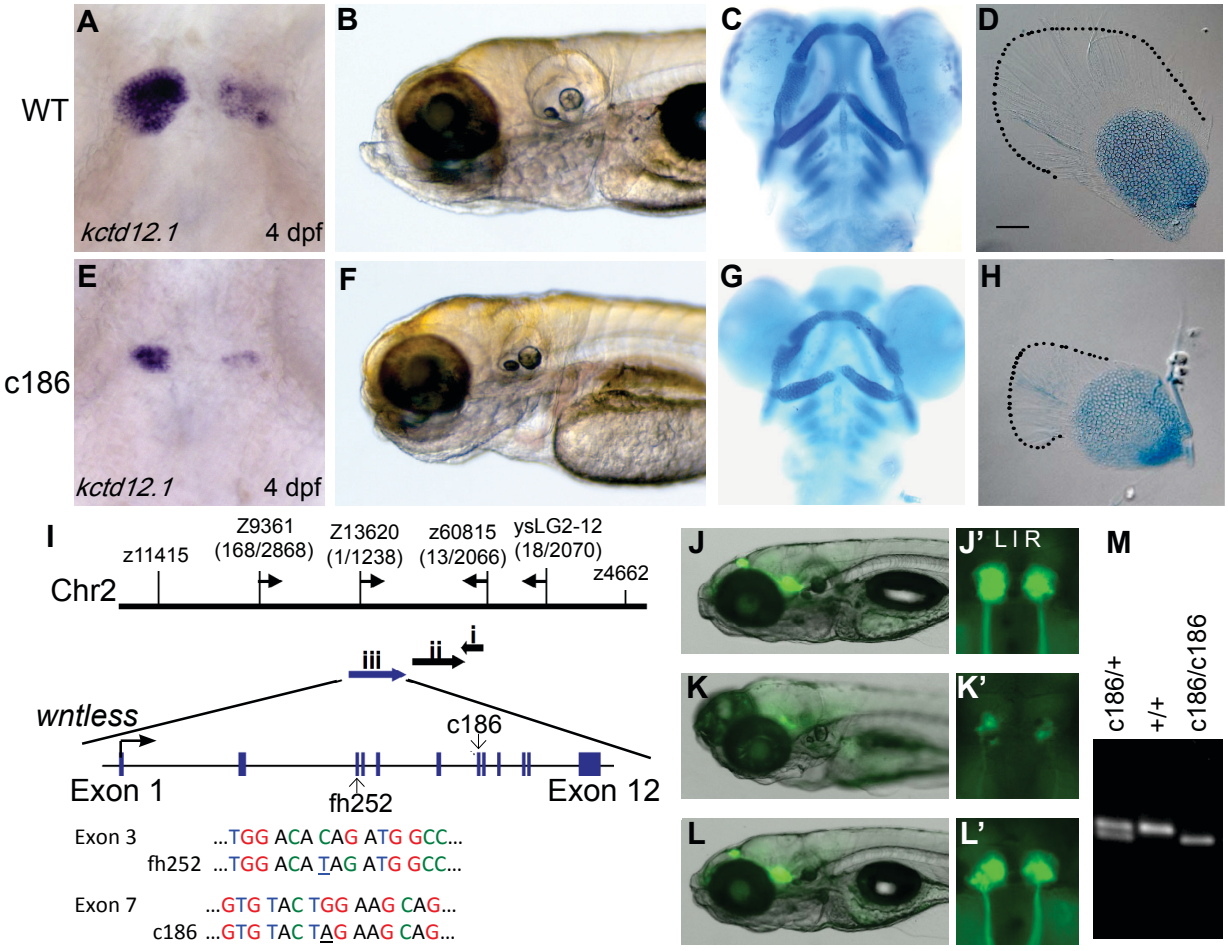
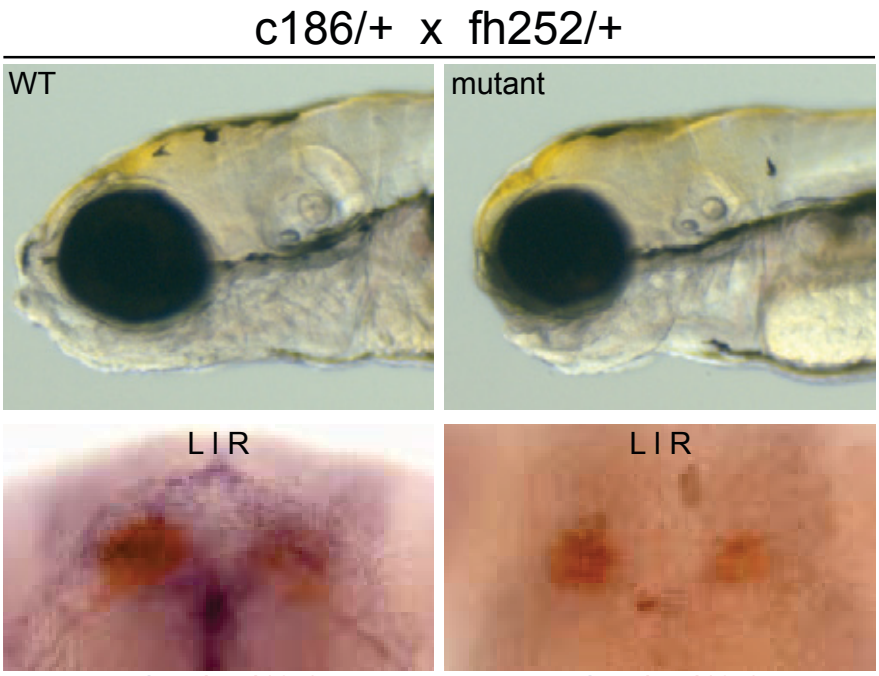

WT
c186
A
B
C
D
E
F
G
H
kctd12.1
4 dpf
kctd12.1
4 dpf
I
z11415
Z9361
(168/2868)
Z13620
(1/1238)
z60815
(13/2066)
ysLG2-12
(18/2070)
z4662
Chr2
iii
ii
i
wntless
c186
Exon 1
fh252
Exon 12
Exon 3 ...TGG ACA CAG ATG GCC...
fh252 ...TGG ACA TAG ATG GCC...
Exon 7 ...GTG TAC TGG AAG CAG...
c186 ...GTG TAC TAG AAG CAG...
J
J' L I R
K
K'
L
L'
M
c186/+
+/+
c186/c186

Fig. 3: c186 and fh252 alleles fail to complement

Transheterozygous (c186/fh252) *wls* mutants can be readily distinguished from WT larvae at 4 dpf and have similar morphological and dorsal habenular phenotypes to mutants homozygous for each allele.


c186/+ x fh252/+
WT
mutant
L I R
L I R
wls + kctd12.1
wls + kctd12.1

and c186 mutants have identical phenotypes and, as expected, the two mutations fail to complement. The resultant transheterozygous embryos are phenotypically indistinguishable from homozygous mutants for each individual allele, indicating that the two mutations result in an equivalent loss of Wls function (Fig. 3). As confirmation that both are null mutations, *wls* transcripts are not detected by RNA *in situ* hybridization in either c186 or fh252 homozygous embryos after late epiboly (Fig. 5D and data not shown). Absence of *wls* transcripts allows mutant embryos to be distinguished from their WT siblings at stages prior to when morphological phenotypes are evident.

Injection of *in vitro* transcribed *wls* mRNA into 1-2 cell zebrafish embryos rescues the mutant phenotypes, including formation of appropriately sized dorsal habenular nuclei and habenular innervation of the midbrain target, the interpeduncular nucleus (Fig. 2J-M). The extent of rescue was also assessed by the size and morphology of the pectoral fins, otic vesicles and jaw, and the presence of a swim bladder (Table 1). To validate rescue of *wls* homozygous mutants, 25 embryos were scored phenotypically and then processed for DNA extraction and genotyping using a derived cleaved amplified polymorphic sequence assay (Fig. 2M, refer to Materials and Methods). Several embryos that were scored as indistinguishable from WT or only mildly affected were confirmed to be homozygous mutants (n=8, Table 2). Although fully rescued mutants developed normal jaws, fins, and swim bladders and lived for several weeks, they did not survive to adulthood, suggesting an essential later role for Wls. Bioinformatic analyses support the presence of a single *wls* gene in the zebrafish genome as in invertebrates and mammals. Regions flanking the zebrafish *wls* gene on chromosome 2 are syntenic with other teleost species (e.g., medaka and tetraodon, Fig. 4). Zebrafish chromosome 6 also shows partial

	Swim Bladder	Pectoral Fins	Otic Vesicles	Jaw	Dorsal Habenulae	Total embryos
Injected	19%	9%	9%	6%	3%	475
Uninjected	39%	27%	27%	27%	26%	494

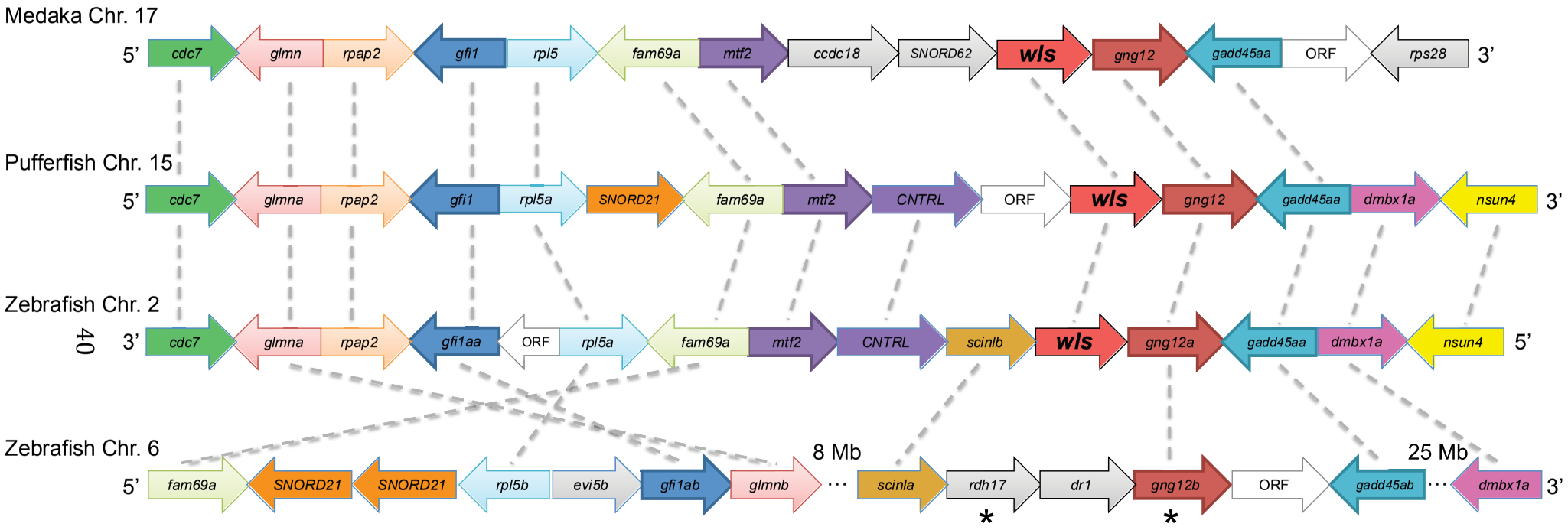
Table 1: Frequency of mutant phenotypes reduced following *wls* mRNA injection.
Seven independent groups of embryos from matings between *wls*^{c186}/+ parents were injected with full-length *wls* mRNA. At 6 dpf, control and injected embryos were assessed for defects in structures associated with the *wls* mutant phenotype (i.e., absence of swim bladder, small pectoral fins and otic vesicles, truncated jaw cartilages and reduced dorsal habenular nuclei).

Extent of rescue	Swim Bladder	Pectoral Fin	Otic Vesicles	Jaw	Dorsal Habenulae
Full	62.5%	62.5%	25%	37.5%	62.5%
Partial	n/a	37.5%	50%	62.5%	37.5%
No	37.5%	0%	25%	0%	0%

Table 2: Phenotypic rescue of homozygous *wls* mutants
Embryos obtained from *wls*^{c186}/+ parents were injected with *wls* RNA. The severity of phenotypes was scored at 7 dpf in the indicated structures. Full rescue is defined as indistinguishable from WT, no rescue represents a morphological defect similar to that observed in *wls* mutants and partial rescue refers to an intermediate phenotype. Larvae were genotyped after phenotypic scoring, and data for 8 confirmed homozygous mutants is shown. Two of the homozygous mutants were completely indistinguishable from WT siblings at the comparable stage.

Fig. 4: Syntenic analysis supports a single zebrafish *wls* gene

The regions surrounding the zebrafish *wls* gene on chromosome (Chr.) 2 are conserved with other teleost species, such as medaka (*Oryzias latipes*) and pufferfish (*Tetraodon nigroviridis*). Zebrafish chromosome 6 also shows partial synteny, but contains multiple insertions, deletions and inversions. DNA sequence homologous to the *wls* gene is not found within the expected region between *scinla* and *gng12a*. Asterisks signify previously unannotated genes and ORF indicate open reading frames of unknown genetic identity.


Medaka Chr. 17
5'
cdc7
glmn
rpap2
gfi1
rpl5
fam69a
mtf2
ccdc18
SNORD62
wls
gng12
gadd45aa
ORF
rps28
3'
Pufferfish Chr. 15
5'
cdc7
glmna
rpap2
gfi1
rpl5a
SNORD21
fam69a
mtf2
CNTRL
ORF
wls
gng12
gadd45aa
dmbx1a
nsun4
3'
Zebrafish Chr. 2
40
3'
cdc7
glmna
rpap2
gfi1aa
ORF
rpl5a
fam69a
mtf2
CNTRL
scinlb
wls
gng12a
gadd45aa
dmbx1a
nsun4
5'
Zebrafish Chr. 6
8 Mb
25 Mb
5'
fam69a
SNORD21
SNORD21
rpl5b
evi5b
gfi1ab
glmnb
scinla
rdh17
dr1
gng12b
ORF
gadd45ab
dmbx1a
3'
*
*

synteny indicative of genomic duplication. However, sequence homology to *wls* is not detected within the duplicated region where the gene is expected to reside (Fig. 4).

Maternal and zygotic *wls* activity

Wnt signaling is necessary for patterning of the germ layers, axis formation and coordinated cell migration during gastrulation (e.g., Harland & Gerhart, 1997; Heisenberg et al., 2000; Kilian et al., 2003; Schier & Talbot et al., 2005). These processes appear normal in zebrafish *wls* mutants, suggesting that maternally deposited *wls* mRNA or protein fulfills the early requirement for Wnt signaling in the developing embryo. Accordingly, maternal *wls* transcripts are present in 100% of the progeny from heterozygous matings (Fig. 5A,B) until approximately 90% epiboly, at which time transcripts are no longer detected in 25% of the embryos (Fig. 5C-D').

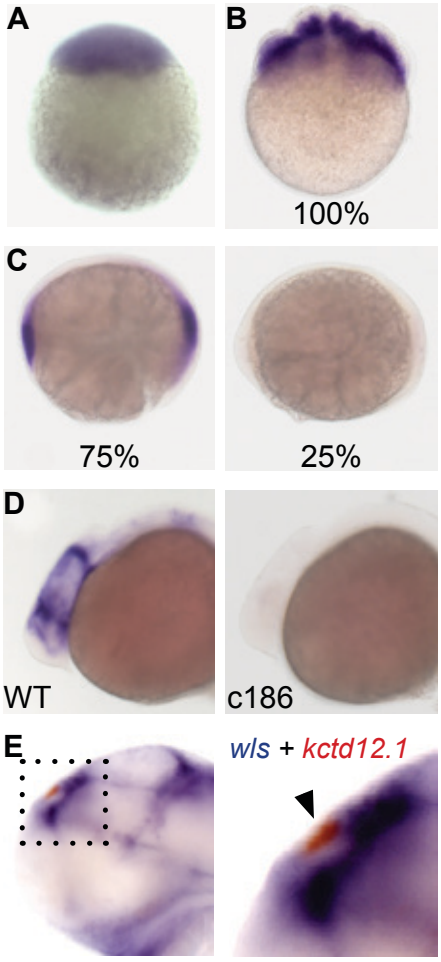
The analysis of zygotic *wls* expression is consistent with the previous findings of Jin et al. (2010), who reported transcripts in the diencephalon, jaw, otic vesicles and fins, the same tissues affected in *wls* mutants (Fig. 5D-E and data not shown). At 48 hpf, *wls* is strongly expressed in the diencephalon, but not within the developing habenulae themselves (arrowheads, Fig. 5E').

Wnt signaling is reduced in zygotic *wls* mutants

Canonical Wnt signaling is known to play an important role in anterior-posterior patterning of the zebrafish brain (Heisenberg et al., 2001; Paridaen et al., 2009). In the mouse, a conditional *wls* mutation produced by a Wnt1-Cre driver causes a decrease in canonical Wnt signaling and a loss of the tectum, cerebellum, tegmentum and choroid

Fig. 5: *wls* is maternally deposited and not expressed in the dorsal habenular nuclei

Maternally derived *wls* transcripts are detected at the (A) 1-cell and (B) 8-cell stage. All embryos derived from heterozygous (wls^{c186}/+) parents have *wls* transcripts; however, by (C) 90% epiboly, they are no longer detected in 25% of the progeny. (D) Expression of *wls* in the diencephalon and midbrain-hindbrain boundary at 24 hpf is not observed in homozygous *wls* mutants. (E) *wls* transcripts (blue) are found in cells surrounding but not within the dorsal habenular nuclei (red, indicated by arrowhead in boxed area shown on right).


A
B
100%
C
75%
25%
D
WT
c186
E
wls + kctd12.1

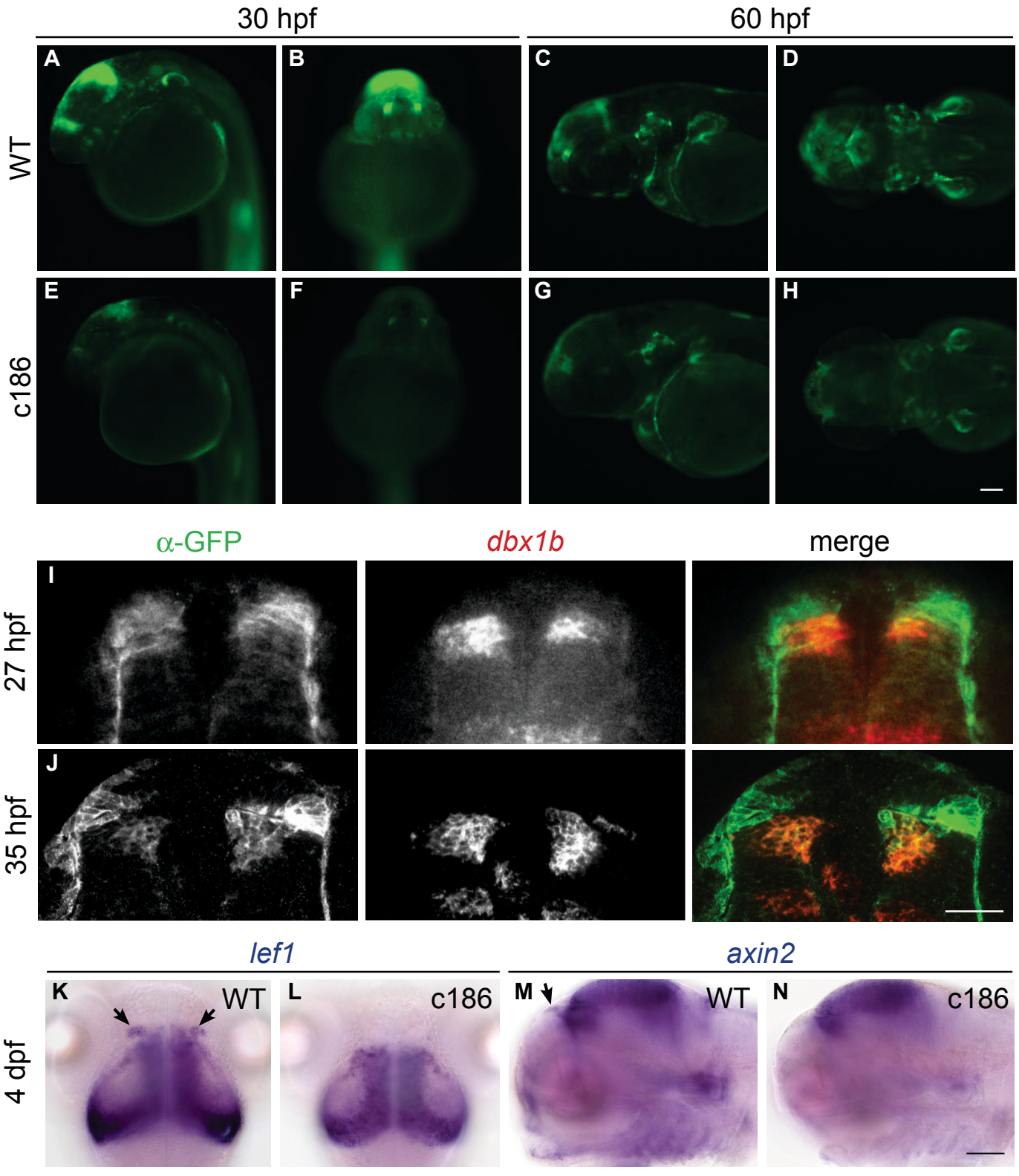
plexus (Carpenter et al., 2010; Fu et al., 2011). To determine whether zebrafish mutants also show reduced activation of the Wnt pathway, we introduced the *wls* mutation into *Tg(7xTCF-Xla.Siam:GFP)*^{ia4}, a transgenic line carrying a reporter of canonical Wnt signaling (Moro et al., 2012). As early as 30 hpf, homozygous mutant embryos bearing the transgenic Wnt reporter exhibit a notable decrease in GFP labeling in the dorsal diencephalon, midbrain-hindbrain boundary and otic vesicles (compare Fig. 6A,B to E,F). By 60 hpf, fluorescence is also diminished in the pectoral fins and jaw (Fig. 6C-D, G-H)

To determine the relationship between Wnt responsive cells and habenular development, we examined expression of the *developing brain homeobox 1b* (*dbx1b*) gene, whose transcripts are highly enriched in the developing habenulae and thalamic progenitors of the mouse (Chatterjee et al., 2014; Quina et al., 2009; Vue et al., 2007) and in proliferating cells of the presumptive dorsal habenulae of zebrafish (Dean et al., 2014). In WT embryos, GFP labeling from the Wnt reporter partially overlaps with *dbx1b* expression at 27 and 35 hpf (Fig. 6I,J)which confirms that canonical Wnt signaling is occurring within the developing dorsal habenulae.

We next examined whether reduced Wnt activity in the brain affected downstream components of the canonical pathway. Expression of the *lef1* and *axin2* genes is known to be activated by Wnt signaling (Holland et al., 2013; Jho et al., 2002). Transcripts for *lef1* were found at near normal levels in the midbrain, but were noticeably reduced in the dorsal diencephalon at 48 hpf and 4 dpf (Fig. 6K,L and data not shown).

Fig. 6: Wnt signaling is reduced in *wls* mutants.

(A-H) The c186 mutation was introduced into a transgenic reporter of canonical Wnt signaling, Tg(7xTCF-Xla.Siam:GFP)^{ia4} (Moro et al., 2012). At 30 hpf, (A,B) WT siblings show robust GFP labeling in the dorsal diencephalon, midbrain-hindbrain boundary and otic vesicles compared to (E,F) c186 mutants (A, E lateral views, B, F frontal views). By 60 hpf, GFP labeling is also found in the pectoral fins and jaw of (C, D) WT embryos and is reduced in (G,H) c186 mutants (C, G lateral views, D, H dorsal views). (I,J) GFP labeling from Tg(7xTCF-Xla.Siam:GFP)^{ia4} colocalizes with *dbx1b* expression in the presumptive habenulae at 27 hpf (single section) and 35 hpf (maximum projection of 15 sections at 0.3 □m) (K,M) WT and (L,N) c186 mutant embryos were assayed for expression of the Wnt-responsive genes (K,L) *lef1* and (M,N) *axin2*. Bilateral *lef1* expression domains in the dorsal diencephalon (arrows in K) are absent in *wls* mutants at 4 dpf, as are *axin2* transcripts in a similar region of the brain (arrow in M). Expression of *axin2* is also reduced in the developing jaw and otic vesicles of (N) *wls* mutants. (K,L dorsal, M,N lateral views). Scale bar = 100 μm for A-H and K-N and 50 μm for I,J.


30 hpf
60 hpf
A
B
C
D
WT
E
F
G
H
c186
α-GFP
dbx1b
merge
I
27 hpf
J
35 hpf
lef1
axin2
K
WT
L
c186
M
WT
N
c186
4 dpf

Similarly, fewer *axin2* transcripts were detected in the dorsal diencephalon, otic vesicles and developing jaw (Fig. 6M,N), regions that are affected in *wls* mutants. The reduction rather than complete loss of Wnt reporter activity, and the presence of Wnt-dependent gene expression outside of the dorsal diencephalon, indicate that Wnt signaling is only partially diminished in the developing nervous system of zygotic *wls* mutants.

Patterning of the brain and habenular L-R asymmetry are intact in *wls* mutants

Despite the reduction in labeling from the canonical Wnt reporter, the morphology and regionalization of the brain is largely normal in *wls* mutant embryos. Anterior-posterior and dorsal-ventral patterns of gene expression are indistinguishable from WT embryos (Fig. 7A). An exception is expression of *neurogenin-1* (*neurog1)* in a small domain in the dorsal diencephalon just ventral and caudal to the habenulae. This domain is significantly reduced or absent in *wls* mutants compared to their WT siblings (arrowheads, Fig. 7A). We assessed whether the lack of *neurog1* expression is responsible for the small dorsal habenulae by examining *neurog1*^{hi1059} homozygous mutants (Golling et al., 2002). The size of the dorsal habenular nuclei is similar in mutant and WT brains (Fig. 8), indicating that while loss of Wls leads to a decrease in *neurog1*-expressing cells in the diencephalon, a reduction in *neurog1* itself does not appear to affect dorsal habenular development.

As Wnt signaling has been shown to influence L-R asymmetry of the dorsal habenulae (Carl et al., 2007; Hüsken et al., 2014) and is important for formation of the ventral habenular nuclei (Beretta et al., 2013), we examined whether these features are

Fig. 7: Defects in brain patterning confined to dorsal diencephalon

(A) Spatially restricted patterns of gene expression in the brains of WT and *wls* mutant larvae at 48 hpf. WT larvae are distinguished from homozygous *wls* mutants by the presence of *wls* transcripts (red), with the exception of *kctd12.1* (red) and *neurog1* double-labeling, where they are distinguished by dorsal habenular size. The *neurog1* expression domain adjacent to the habenulae (arrowhead in WT) is reduced in *wls* mutants . (B) Gene expression in the habenular region. The dorsal habenulae of *wls* mutants show reduced *f-spondin* and *ano2* expression but, despite their smaller size, L-R asymmetric gene expression (*nrp1, kctd12.2*, *vachtb*) is maintained (right dorsal nucleus indicated by arrow for *vachtb*). Expression of *vachtb* and *aoc1* in the ventral habenulae (arrowheads in WT) is largely absent in *wls* mutants. The parapineal is positioned to the left of the pineal analage, as determined by *otx5* expression, in WT and *wls* mutant siblings. Scale bars = 100 μm.

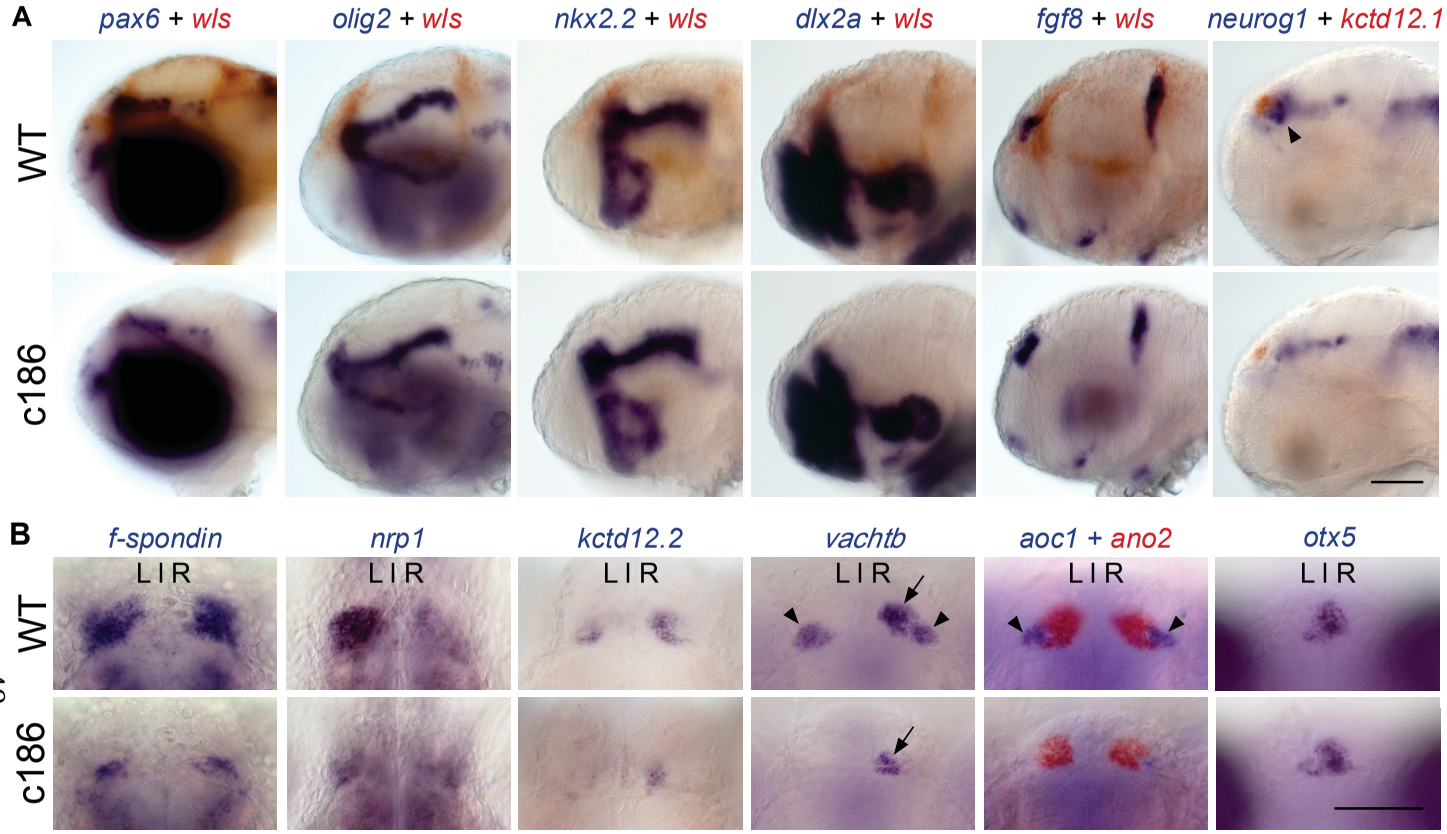
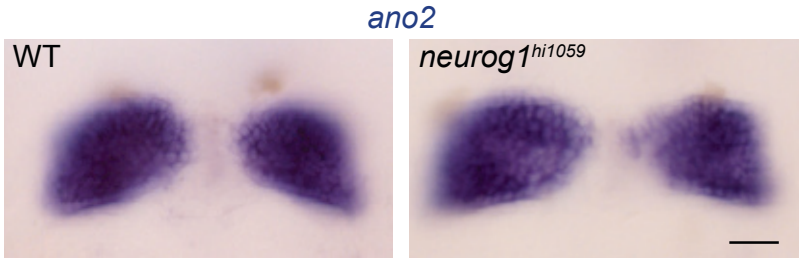

A
pax6 + wls
olig2 + wls
nkx2.2 + wls
dlx2a + wls
fgf8 + wls
neurog1 + kctd12.1
WT
c186
B
f-spondin
nrp1
kctd12.2
vachtb
aoc1 + ano2
otx5
LIR
WT
c186

Fig. 8: Dorsal habenular nuclei develop normally in *neurogenin-1* mutants

WT and homozygous *neurog1*^{hi1059} mutants show similar expression of *ano2* in the dorsal habenular nuclei. The *neurog1*^{hi1059} allele is due to a retroviral insertion (Golling et al., 2002) and, although not confirmed to be a null mutation, when homozygous, it disrupts formation of sensory neurons (McGraw et al., 2008).


ano2
WT
neurog1hi1059

disrupted in the absence of Wls. The bilateral expression domains of genes such as *f-spondin* and *ano2* are smaller in *wls* mutants compared to WT siblings (Fig. 7B).

Although the dorsal habenulae are reduced in size, and consistent with the initial results on *kctd 12.1* (Fig. 2A,E), the asymmetric expression patterns of genes showing either a left- or right-sided bias are preserved (Fig. 7B). Leftward migration of the parapineal, a structure known to influence habenular laterality (Gamse et al., 2003, Concha et al., 2000), also appears unperturbed in *wls* mutants, as determined by expression of *otx5* in the pineal and parapineal (Fig. 7B). However, as in *tcf7l2* mutants (Beretta et al., 2013), the ventral habenular nuclei of *wls* mutants are absent (Fig. 7B). These findings indicate that the functions of Wnt signaling in L-R asymmetry of the epithalamus and formation of the ventral habenular nuclei are separable.

Habenular precursor populations are affected in *wls* mutants

The small size of the dorsal habenulae of *wls* mutants could result from abnormal neurogenesis, as it is well known that Wnt signaling influences this process (Ciani & Salinas, 2005; Ille & Sommer, 2005). To assess cell death and cell proliferation we performed terminal deoxynucleotidyl transferase dUTP nick end labeling **(**TUNEL) to detect apoptotic cells and immunolabeing using a phospho-Histone H3 (pH3) antibody that recognizes mitotic cells, at stages when the developing habenulae could be distinguished in *TgBAC(dbx1b:GFP)* embryos. An equivalent low number of TUNEL positive cells (0-2 cells, data not shown) were detected at 27 hpf in the GFP labeled habenular region of c186 homozygotes (n=6) as in their WT siblings (n=12). At 48 hpf,

actively dividing $pH3^+$ cells were found in equivalent numbers relative to the volume of the *dbx1b:GFP* habenular domain in mutant and WT embryos (Fig. 9). Therefore, neither increased cell death, nor disruption of proliferation appear responsible for the reduced dorsal habenulae of *wls* mutants.

An alternative role for Wls-dependent Wnt signaling could be in the specification and/or maintenance of habenular progenitors. To test for this, we examined *dbx1b* expression at 23 hpf, when transcripts are first detected in the dorsal diencephalon of the majority (57%) of WT embryos. At this stage, *dbx1b* transcripts were not detected in the same brain region of *wls* mutants (Fig. 10A,A'; Table 3). By 26 hpf, when almost all WT siblings show robust *dbx1b* expression in the developing habenulae (92%; Table 3), only a few $dbx1b^+$ cells were observed in 27% of *wls* mutants (Fig. 10B,B'; Table 3). These findings suggest that there is a temporal delay in the development of the $dbx1b^+$ habenular progenitor cell population.

Previously, the chemokine (C-X-C motif) receptor 4b (*cxcr4b*) gene was described as another marker of habenular progenitor cells or early habenular neurons (Roussigné et al., 2009). Since *cxcr4b* is expressed in a similar pattern as *neurog-1* (Roussigné et al., 2009), a global marker of neural progenitors whose expression is reduced in *wls* mutants (Fig. 7A), we hypothesized that the small dorsal habenulae could also result from a lack of $cxcr4b^+$ cells. From 27 hpf onward, homozygous *wls* mutants show characteristic defects in both the *dbx1b* and *cxcr4b*-expressing habenular progenitor populations, while expression of these genes is unperturbed in other regions of the brain (Fig. 11 and data not shown). Compared to WT, fewer $cxcr4b^+$ cells were found in the diencephalon of *wls* mutants at 27 hpf and 35 hpf (arrowheads Fig. 11A-B'). As early as

27 hpf, the bilateral *dbx1b*$^{+}$ habenular domains are noticeably smaller (Fig. 11E-F',H-I') and, by 48 hpf, the presumptive habenulae of *wls* mutants, as defined by *cxcr4b* (Fig. 11C,C') and *dbx1b* (Fig. 11G,G',J,J') expression, are significantly reduced in size. Thus, a deficit in two progenitor populations accounts for the small dorsal habenular nuclei of *wls* mutant zebrafish.

Fig. 9: Cells proliferate within the developing habenular region of *wls* mutants

(A) Immunolabeling was performed at 48 hpf on *TgBAC(dbx1b:GFP)* embryos to visualize $pH3^+$ mitotic nuclei within the developing habenulae. Habenular volume was calculated by generating a 3D virtual representation of the *dbx1b:GFP* labeled domain (green) using the surfaces function of Imaris image processing software (Bitplane). $pH3^+$ cells unambiguously located within this domain (red) were identified computationally using the spots function of Imaris. Two representative examples of *wls*^{c186} homozygous mutants and WT siblings are shown post-processing. Scale bar = 20 μm. (B) Although the number of $pH3^+$ cells and volume of the developing habenulae (value of y axis X 10^4 μm^3) differed between mutant (n= 11) and WT (n= 15) embryos (two-tailed t-test, $p=6.29 \times 10^{-8}$ and $p=4.21 \times 10^{-12}$, respectively), when normalized for volume, the number of mitotic cells within the *dbx1b:GFP* habenular domain was not significantly different between the two groups (p=0.12).

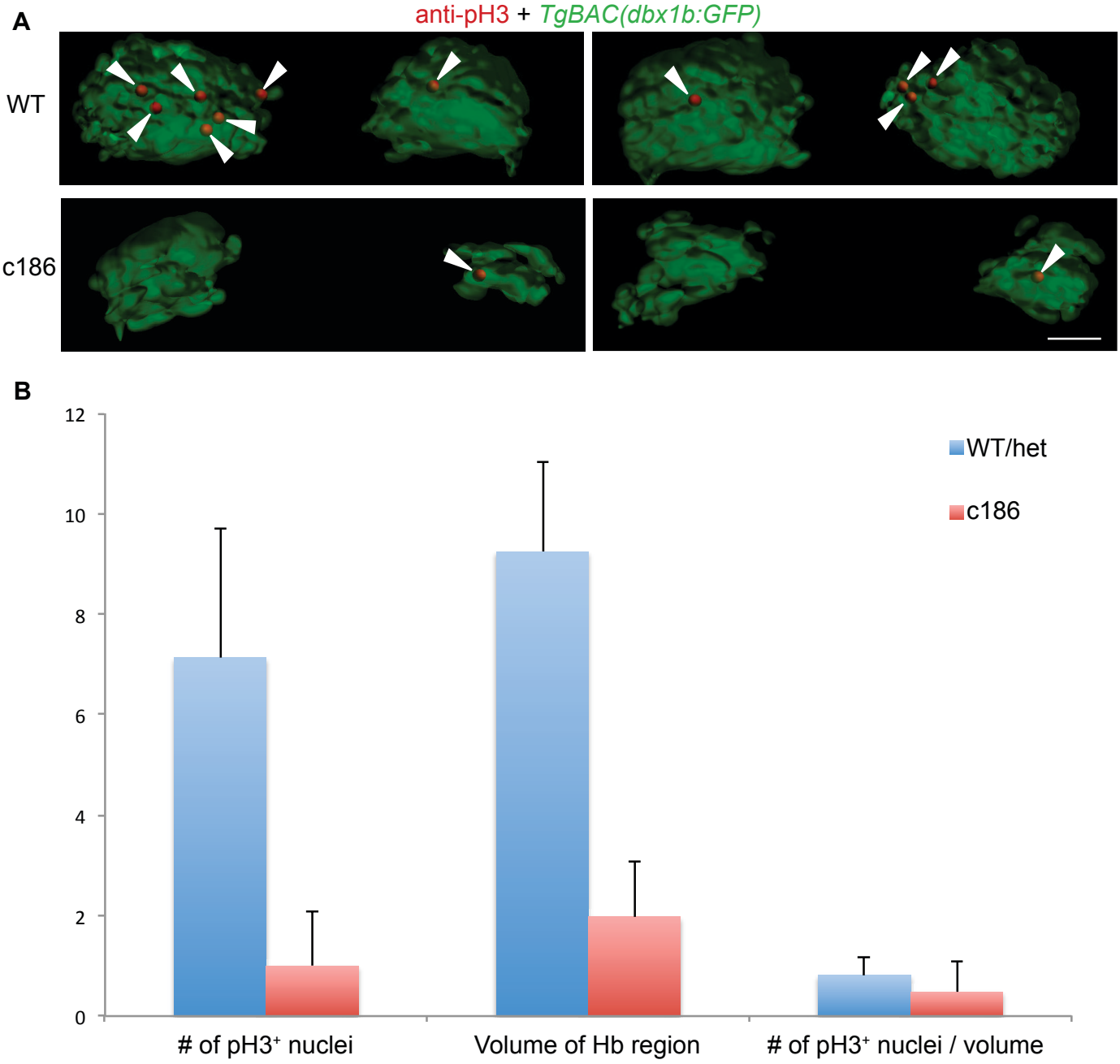
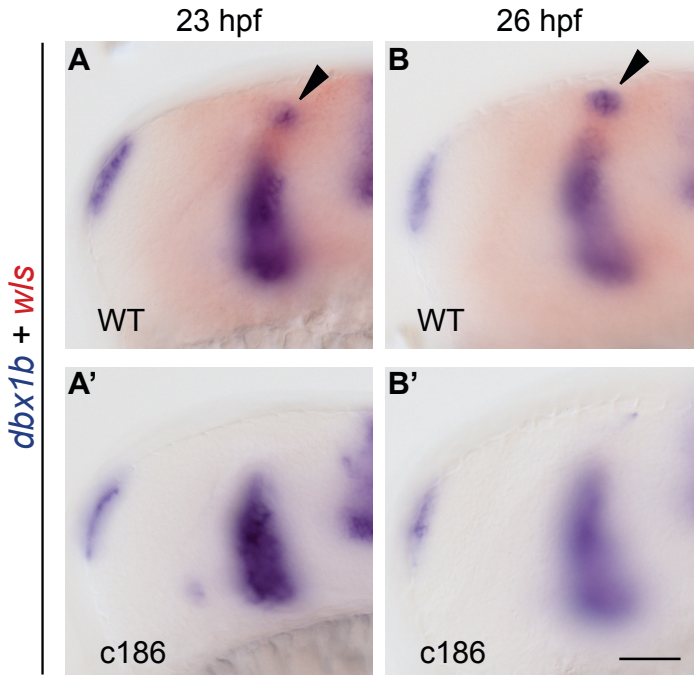

A
anti-pH3 + TgBAC(dbx1b:GFP)
WT
c186
B
12
10
8
6
4
2
0
WT/het
c186
# of pH3⁺ nuclei
Volume of Hb region
# of pH3⁺ nuclei / volume

Fig. 10: *dbx1b*$^{+}$ expression in the developing habenulae is delayed in wls mutants

Lateral view of *dbx1b* transcripts in the dorsal diencephalon of (A-A') 23 hpf and (B-B') 26 hpf WT and c186 embryos. At these early stages, *wls* expression (red) distinguishes WT siblings from homozygous mutants. Scale bar = 50 μm.


23 hpf
26 hpf
A
B
WT
WT
A'
B'
c186
c186
dbx1b + wls

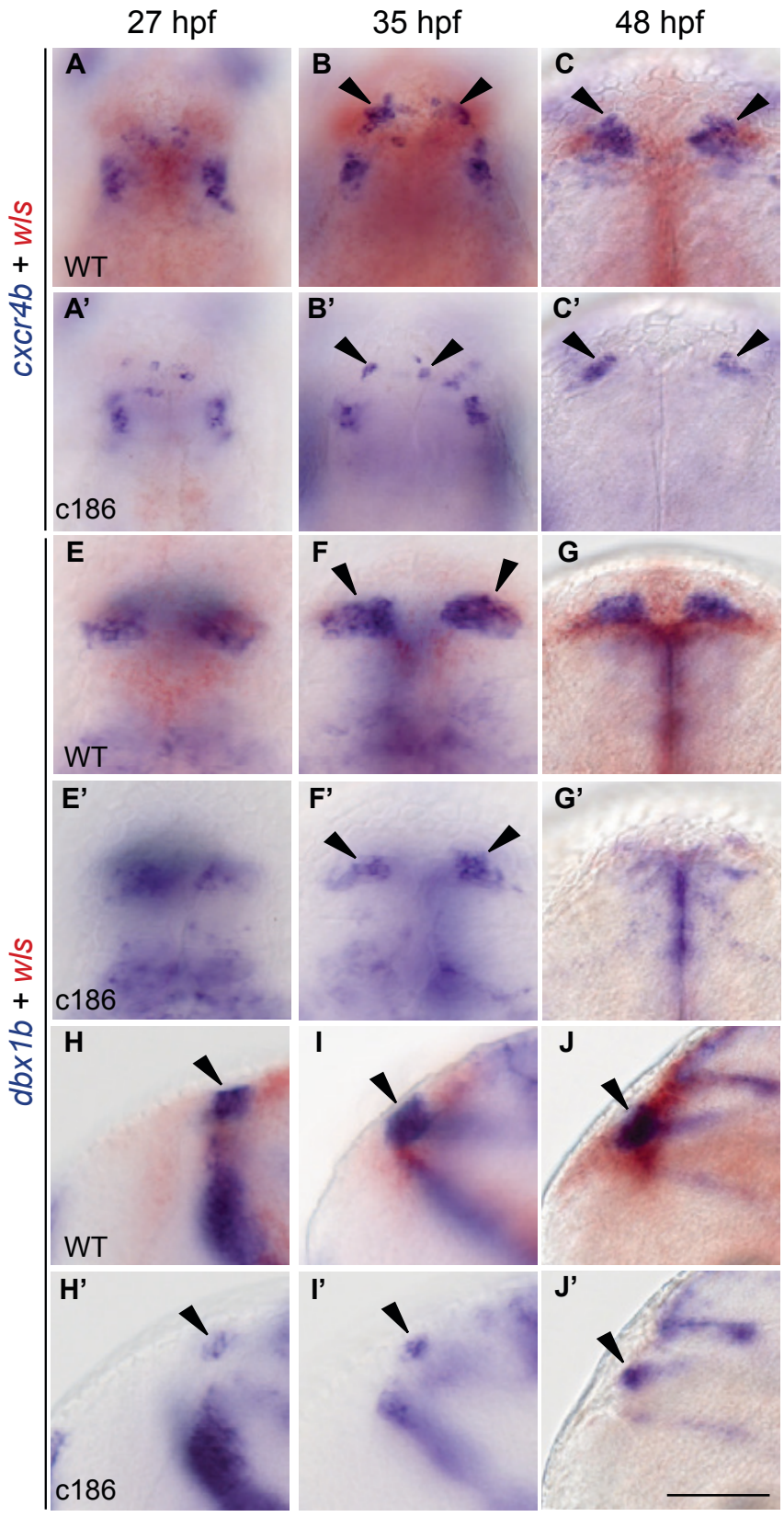
Presence of *dbx1b*$^{+}$ cells	**WT**	***wls* mutants**
23 hpf	57% (n= 19)	0% (n= 5)
26 hpf	92% (n= 13)	27% (n= 11)

Table 3: Expression of dbx1b is delayed in the developing habenulae of wls mutants.

Percent of embryos with dbx1b expression in the habenular region at 23 and 26 hpf for wlsc186 mutants and their WT siblings.

Fig. 11: Habenular precursor populations are reduced in *wls* mutants

(A-C') Dorsal views of *cxcr4b* expression in the diencephalon of WT and *wls* homozygous mutant embryos. Dorsal (E-G') and lateral (H-J') views of *dbx1b* expression in WT and *wls* mutant embryos. *wls* transcripts (red) were used to distinguish WT siblings from homozygous mutants. Scale bar = 100 μm.


27 hpf
35 hpf
48 hpf
cxcr4b + wls
dbx1b + wls
A
B
C
WT
A'
B'
C'
c186
E
F
G
WT
E'
F'
G'
c186
H
I
J
WT
H'
I'
J'
c186

DISCUSSION

During the course of a mutagenesis screen to identify genes that regulate habenular development, we isolated a mutation that results in small dorsal habenular nuclei and localized it to the zebrafish homolog of the *wntless* gene. The Wntless protein is essential for the transport of Wnts to the cell surface in both invertebrates and mammals (Das et al., 2012). Our study now implicates Wls in proper formation of the habenular region of the zebrafish forebrain. Zebrafish *wls* is expressed in the developing dorsal diencephalon in domains directly adjacent to but not within the habenulae. Characterization of the *wls* mutant phenotype supports distinct requirements for Wnt signaling in habenular development: an early function in establishing L-R asymmetry of the dorsal nuclei (Carl et al., 2007; Hüsken et al., 2014) and later roles in formation of the ventral habenular nuclei (Beretta et al., 2013) and in the generation of a complete repertoire of dorsal habenular progenitors.

Identification of a zebrafish Wntless homolog

A *wls* homolog was previously isolated from the zebrafish genome on the basis of the high amino acid identity of its predicted protein with the human and mouse Wntless proteins (Jin et al., 2010). The zebrafish protein is 78% identical to human WLS isoform 1 and 76% to mouse isoform a, but only 42% identical to the Wls isoform B of *Drosophila*. In addition to sequence conservation at the amino acid level, we determined that the predicted zebrafish protein contains a tyrosine-glutamate-glycine-leucine (YEGL) motif that is required for retromer-dependent recycling from the cell membrane

(Gasnereau et al., 2011). Analyses of sequence homology and syntenic chromosomal regions indicate that this is the sole Wls gene in the zebrafish genome.

The initial ENU induced c186 mutation and the fh252 mutation identified by TILLING contain premature stop codons in exons 7 and 3, respectively. Both are loss-of-function mutations since aberrant, zygotically-derived *wls* mRNA is not detected in homozygous mutants and is most likely a target of nonsense-mediated decay.

Conserved aspects of the zebrafish *wls* mutant phenotype

Zebrafish *wls* mutants show defects in a variety of tissues, including the jaw and pectoral fin cartilages and the otic vesicles. To further explore the jaw phenotype seen in the *wls* mutants, I provided fixed embryos to a collaborator (Rochard et al., 2016). They identified Wnts 5b and 9 as responsible for the non-canonical signaling that drives cell intercalation necessary for palate extension. Previous studies using a morpholino to deplete zebrafish Wls also reported irregularities in jaw and otic vesicle development (Jin et al., 2010; Wu et al., 2015). In addition to the jaw, severe phenotypes such as small heads and cardiac edema were described (Jin et al., 2010) that are not observed in *wls* homozygous mutants, indicative of non-specific morpholino toxicity. Conditional mouse mutants generated with a Wnt1:Cre driver show craniofacial abnormalities reminiscent of the jaw phenotype of *wls* mutant zebrafish (Carpenter et al., 2010; Fu et al., 2011). A reduction in limb structures, analogous to the fish pectoral fin, was observed in conditional *wls* mutant mice produced with a Prx1:Cre driver (Zhu et al., 2012). Defects in the ear and habenular region of the brain have not yet been reported in conditional *wls* mutant mice; however, the development of these tissues may depend on Wls derived

from other sources than *wnt1*- or *prx1*-expressing cells. Thus, the use of additional Cre driver lines may reveal alterations in analagous structures in zebrafish and mice.

Does Wntless mediate all Wnt signaling in zebrafish?

Given the role of Wls in Wnt signaling, we expected to find numerous Wnt-related defects in zebrafish homozygous mutants. However, zebrafish *wls* mutants undergo surprisingly normal early development. This contrasts with *wnt5b* and *wnt11* mutants, which have severe abnormalities in convergence-extension during axis formation (Heisenberg et al., 2000, Marlow et al., 2004) or other Wnt signaling mutants that show disruptions in brain patterning and organogenesis (e.g., Heisenberg et al., 2001; Hikasa & Sokol, 2013; Lee et al., 2006, Lin & Xu, 2009, Matsui et al., 2005, Paridaen et al., 2009; Poulain & Ober, 2011). Furthermore, expression of genes downstream of canonical Wnt signaling is only notably affected in the dorsal diencephalon of *wls* mutants and labeling from a transgenic reporter of canonical Wnt signaling is also diminished rather than absent and low levels of GFP labeling persist in mutants as late as 6 dpf.

A likely explanation for the relatively normal early development of *wls* mutants and the persistence of Wnt reporter activity is the presence of maternally deposited mRNA and protein. Given that maternally deposited WT *wls* mRNA can persist until 90% epiboly (8-9 hpf), Wls protein could be present throughout much of early embryonic development in zygotic mutants. Indeed, a recent study confirms the presence of maternally derived Wls protein in zebrafish embryos, at levels sufficient for embryos injected with *wls* antisense morpholinos to develop normally at early stages (Wu et al., 2015). Maternal deposition could also account for the difference in phenotypic severity

between zebrafish *wls* homozygotes and *wls* mutant mice (Carpenter et al., 2010; Fu et al., 2009) or *Drosophila* embryos derived from *wls* mutant germline clones (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). The precise roles of maternally provided Wls need to be rigorously tested in zebrafish by eliminating the maternal contribution and generating a maternal-zygotic mutant.

An alternative possibility to account for the relatively mild phenotype of *wls* homozygotes is that some Wnts may be released in a Wls-independent manner. A subset of genes that are positively regulated by and highly sensitive to Wnt signaling are transcribed at low levels in Drosophila lacking *wls* function (Banziger et al., 2006; Goodman et al., 2006), suggesting that in the absence of Wls, some Wnt signals can still be received by Wnt-responsive cells. Wnt family members have also been identified that do not require the acyltransferase Porcupine, which, unless they are lipid modified by a different mechanism, would allow for their secretion independent of Wls (Chen et al., 2012; Richards et al., 2014). WntD, for example, is neither lipidated nor requires Wls for its intracellular trafficking and secretion from Drosophila cells (Ching et al., 2008). Depletion of zebrafish Wls using an antisense morpholino further argues for a differential requirement for this protein in Wnt trafficking, as membrane localization of fluorescently tagged Wnt5b is severely disrupted, whereas Wnt11 persists at the cell membrane, indicative of active secretion (Wu et al., 2015). Moreover, not all *wnt* genes show co-expression with *wls* in the zebrafish embryo (Kuan and Halpern, unpublished observations), suggesting that alternative strategies must exist for trafficking of the Wnts that they encode. A recent study suggests that a protein homologous to *C. elegans*

Unc119-c may perform similar functions to Wls as a Wnt chaperone in zebrafish (Toyama et al., 2013).

Multiple roles for Wnt signaling in habenular development

Several features of habenular development have been attributed to the canonical Wnt signaling pathway. Analyses of *axin1* and *tcf7l2* mutants indicate that Wnt signaling is important for directional asymmetry of the dorsal habenulae (Carl et al., 2007; Hüsken et al. 2014). It is therefore surprising that L-R differences between the dorsal habenulae are intact in *wls* mutants.

Another demonstrated function of the Wnt signaling pathway is in the formation of the ventral habenulae, which are lacking in *tcf7l2* mutants (Beretta et al., 2013). Markers of the ventral habenulae are also undetected or their expression greatly diminished in *wls* mutants. Thus, while L-R asymmetry of the dorsal nuclei and formation of the ventral nuclei are both reliant on Wnt signaling (Beretta et al., 2013**;** Hüsken et al, 2014), analysis of the *wls* mutant phenotype demonstrates that these features of habenular development are independently regulated. The most parsimonious explanation is that there are temporally distinct roles for canonical Wnt signaling, with an early function in L-R asymmetry and a later one in the formation of the ventral habenulae. L-R differences in the developing brain are found as early as 18 hpf, as evidenced by expression of *lefty1, cyclops*, and *pitx2* on the left side of the dorsal diencephalon (Concha et al., 2000; Rebagliati et al., 1998; Sampath et al., 1998; Thisse & Thisse, 1999). In contrast, ventral habenular precursors are thought to be present at 2 dpf (Beretta et al., 2013)**.** Because of this difference in timing, we propose that maternally

derived Wls activity plays a role in the establishment of directional asymmetry and determination of dorsal habenular L-R identity, whereas zygotic Wls is necessary for specification of the ventral nuclei.

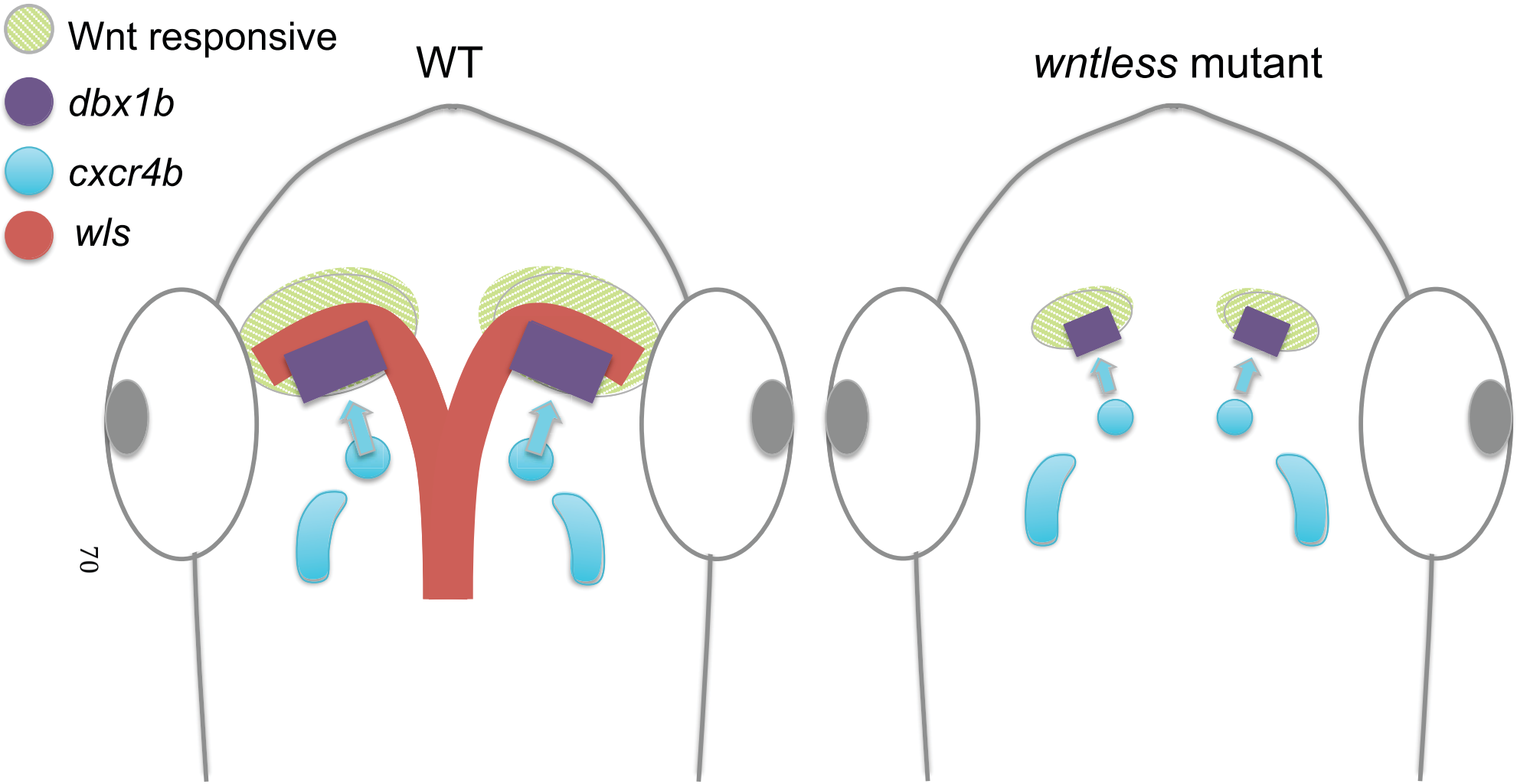
Characterization of the *wls* phenotype reveals an additional role for Wnt signaling in the regulation of dorsal habenular precursor populations, as depicted schematically in Fig. 12. As early as 27 hpf, Wnt responsive cells are found within the *dbx1b* domain, suggesting that proper development of dorsal habenular progenitors is dependent on Wnt signaling. Attenuated Wnt signaling in the diencephalon may cause the delay in the production of and ultimate reduction in numbers of *dbx1b*$^{+}$ dorsal habenular progenitors. By 35 hpf, there are notably fewer *cxcr4b*-expressing cells in the developing habenulae, though they are present more caudally in the diencephalon. Given the known role of Cxcr4b in cell migration of the lateral line primordium and olfactory placodes (Miyasaka et al., 2007; Venkiteswaran et al., 2013), we suspect that the *cxcr4b*-expressing habenula progenitors migrate anteriorly to join the newly forming *dbx1b*-expressing dorsal habenulae. Recruitment of fewer *cxcr4b*-expressing cells to the *dbx1b*-expressing presumptive habenulae could further reduce the mutant dorsal habenular nuclei. Intriguingly, mutations in *axin1* or *tcf7l2* do not cause a reduction in the size of the dorsal habenulae (Carl et al., 2007; Hüsken et al., 2014) as observed in *wls* mutants. Proper formation of the *dbx1b* presumptive dorsal habenulae and recruitment of the *cxcr4b*-expressing habenular progenitors may, therefore, rely on other downstream components in the Wnt signaling pathway.

Reduced habenular size has been implicated in bipolar disorder and major depressive disorder (Ranft et al., 2010; Savitz et al., 2011). Intriguingly, lithium chloride,

a common treatment for bipolar disorder, is both a Wnt agonist (Klein & Melton, 1996) and increases habenular volume in bipolar patients (Savitz et al., 2011). While it is premature to propose a causal relationship between habenular size and psychiatric disorders, *wls* mutants provide a valuable model to explore the multiple roles of Wnt signaling in the development and ultimately the function of the habenular region of the brain.

Fig. 12: Role of Wntless in dorsal habenular development.

Schematic model whereby Wls-dependent canonical Wnt signaling influences formation of the *dbx1b*-expressing presumptive dorsal habenulae and the subsequent recruitment of *cxcr4b*-expressing progenitors. Diminished Wnt signaling in homozygous *wls* mutants is proposed to reduce the *dbx1b*-expressing habenular domain and migration of the *cxcr4b*$^{+}$ population to the dorsal habenulae.


Wnt responsive
dbx1b
cxcr4b
wls
WT
wntless mutant

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Chapter 3: Convergence of Signaling Pathways Underlying Habenular Formation and Axon Outgrowth

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ABSTRACT

The habenular nuclei are a conserved integrating center in the vertebrate dorsal diencephalon that modulate diverse behaviors. Despite their importance, our understanding of habenular development is incomplete. Time-lapse imaging and fate mapping demonstrate that the dorsal habenulae (dHb) of zebrafish are derived from *dbx1b*-expressing (*dbx1b*$^{+}$) progenitors, which transition into *cxcr4b*-expressing neuronal precursors. These precursors give rise to differentiated neurons whose axons innervate the midbrain interpeduncular nucleus (IPN). Formation of the *dbx1b*$^{+}$ progenitor population relies on the activity of the Shh, Wnt and Fgf signaling pathways. Wnt and Fgf function additively to generate dHb progenitors. Surprisingly, Wnt signaling also negatively regulates expression of *fgf8a*. Moreover, the Wnt and Fgf pathways have opposing roles in transcriptional regulation of components of the Cxcr4-chemokine signaling pathway. The chemokine pathway, in turn, directs the posterior outgrowth of dHb efferents to the IPN and, when disrupted, results in ectopic, anteriorly directed axonal projections. The results define a signaling network underlying the generation of dHb neurons and connectivity with their midbrain target.

INTRODUCTION

The habenulo-interpeduncular (Hb-IPN) conduction system is a conserved feature of the vertebrate brain that modulates a variety of processes such as sleep, fear/anxiety, pain, learning, feeding, reproduction and reward (Sutherland, 1982; Benarroch, 2015; Namboodiri et al., 2016), and has been associated with mood disorders (Ranft et al., 2010; Savitz et al., 2011; Lawson et al., 2016) and addiction (Fowler et al., 2011; Baldwin et al., 2011). Recent attention has focused on the association of the Hb-IPN circuitry with nicotine dependence and withdrawal symptoms (Antolin-Fontes et al., 2015; Salas et al., 2009; Zhao-Shea et al., 2013; Pang et al., 2016) and the discovery that, in zebrafish and other non-mammalian species, the bilaterally paired dorsal habenulae (dHb; equivalent to the medial habenulae [mHb] of mammals, Aizawa et al., 2011) show prominent L-R differences in their size, organization, molecular properties and connectivity (Braitenberg et al., 1970; Concha & Wilson, 2001; Duboué & Halpern, in press). Although accumulating evidence reveals the importance and diverse roles of the habenular nuclei, our understanding of their development is incomplete.

The Hedgehog (Hh), Wnt, and fibroblast growth factor (Fgf) signaling pathways have all been implicated in early habenular development. However, there are conflicting reports on whether Hh signaling antagonizes or promotes formation of the dHb (Chatterjee et al., 2014; Halluin et al., 2016). Mutations in the zebrafish *wntless* (*wls*) gene, which encodes a protein essential for Wnt secretion, or in the *fgf8a* gene, result in small dHb (Regan et al., 2009; Kuan et al., 2015). The small size of the dHb in *wls* and *fgf8a* mutant zebrafish is likely due to a reduction in dHb progenitors (Kuan et al., 2015; Dean et al., 2014), which are characterized by their expression of the *developing brain*

homeobox 1b (*dbx1b*) gene (Dean et al., 2014). The *dbx1* gene was first identified as a marker of habenular progenitors in mice (Vue et al., 2007). In zebrafish, there are two homologs, *dbx1a* and *dbx1b,* but only *dbx1b* is expressed in dHb progenitors (Dean et al., 2014). Transcripts for the *C-X-C chemokine receptor type 4b* (*cxcr4b*) gene are also presumed to localize to habenular progenitors or newly born neurons (Roussigné et al., 2009; Halluin et al., 2016). However, the relationship between the cells that express *dbx1b* and those that express genes encoding components of the Cxcr4-chemokine pathway is unresolved.

Through long-term lineage experiments, we now demonstrate that *dbx1b*$^{+}$ progenitors give rise to all neurons in the dHb of adult zebrafish. During larval development these progenitors undergo a transition during which they express both *dbx1b* and *cxcr4b*, then downregulate *dbx1b* and differentiate into *cxcr4b* expressing neural precursors and, ultimately, dHb neurons. Hh, Wnt and Fgf signaling pathways are required for formation of *dbx1b*$^{+}$ progenitors. Analyses of zebrafish mutants indicate that Hh signaling is upstream or independent of Wnt signaling, and that Wnt activity restricts the domain of Fgf signaling. Wnt and Fgf signals are also necessary to delimit the expression domains of genes encoding Cxcr4-chemokine pathway components. We discovered that disruption of chemokine signaling perturbs the posteriorly directed outgrowth of dHb efferents, resulting in ectopic axonal projections. Thus, the action of multiple signaling pathways, through a complex regulatory network, promotes the generation and connectivity of habenular neurons.

MATERIALS AND METHODS

Zebrafish husbandry

Zebrafish were kept on a 14:10 light:dark cycle at 27°C. Embryos were raised in system water or treated with 0.003% phenylthiourea (PTU). The AB wild type strain (Walker, 1999) and lines bearing the *wls*^{c186} (Kuan et al., 2015), *fgf8*^{ti282a} (Brand et al., 1996), *cxcr4b*^{t26035} (Knaut et al., 2003) and *smoothened*^{hi1640Tg} (Chen et al., 2001) mutations were used. Homozygous *wls*^{c186} mutants were genotyped as embryos or identified as larvae by morphological criteria (Kuan et al., 2015). Embryos and larvae homozygous for the *cxcr4b* allele were genotyped using published primers (Miyasaka et al., 2007). Transgenic lines used in this study include *TgBAC(gng8:Eco.NfsB-2A-CAAX-GFP)* (deCarvalho et al., 2013), *TgBAC(dbx1b:Cre-mCherry)* (Koyama et al., 2011), *TgBAC(cxcr4b:nls-dTomato)* (Doná et al., 2013), *TgBAC(dbx1b:EGFP)* (Kinkhabwala et al., 2011), *Tg(hsp70:dkk1-GFP)* (Stoick-Cooper et al., 2007), *TgBAC(cxcr4b:cxcr4b-mKate2-IRES-GFP-CaaX)* (Venkiteswaran et al., 2013), *TgBAC(dbx1b:tdTomato-nls)* (Satou et al., 2012), *Tg(HuC:H2B-GCamP6S)* (Vladimirov et al., 2014) and *Tg(β-actin:loxP-hmgb1-eCFP-loxP-H2B-mCherry)* (M. Parsons, unpublished). *TgBAC(gng8:Eco.NfsB-2A-CAAX-GFP)* is herein referred to as *Tg(gng8:CAAX-GFP)*. Zebrafish maintenance and experimental procedures were performed according to protocols approved by the Carnegie Institutional Animal Care and Use Committee.

RNA in situ hybridization and immunofluorescence

Colorimetric and fluorescent *in situ* hybridization were performed according to previously described methods (deCarvalho et al., 2013). RNA probes were synthesized for *dbx1b* (Gribble et al., 2007), *etv5b*, *ackr3b, cxcr4b*, *wls* (Thisse et al., 2001), *fgf8* (Fürthauer et al., 1997), *ano2* (deCarvalho et al., 2014), *dusp6* (Tsang et al., 2004), *cxcl12a* (Doitsidou et al., 2002), *cxcl12b* (Thisse & Thisse, 2005), and *elavl3* (Kim et al., 1996). GFP was detected using rabbit antisera (Torrey Pines Biolabs, Cat. #TP401) and a Cy3-conjugated AffiniPure anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, Cat. #111-165-144). Immunolabeling was performed as described (deCarvalho et al., 2013).

Microscopy and image analysis

An AxioCam HRc camera mounted on a Zeiss Axioskop was used to obtain bright field images. Fluorescent images were captured on an upright or inverted Leica SP5 confocal microscope fitted with a 25x, NA=0.95 water immersion objective. Fixed and live embryos were mounted in 1.2% low-melt agarose (Seaplaque, Lonza) in 1X phosphate buffered saline (PBS) or fish system water with 200 mg/L tricaine (Sigma), respectively. Sectioning and imaging of adult brains was performed as previously described (deCarvalho et al., 2014). Confocal images were analyzed using IMARIS software (Bitplane).

Time-lapse imaging

Embryos were sedated in 165 mg/L tricaine in 1X PTU and were mounted in 1% low-melt agarose in a glass bottom dish (WillCo Wells). These tricaine and agarose concentrations were shown to reduce developmental defects in long-term imaging experiments (Kaufmann et al., 2012). Time-lapse imaging was performed on a Leica SP5 inverted confocal microscope. Z-stacks of the dorsal embryonic brain (~100-300 μm, step size of 1 μm) were acquired at 15-minute intervals. Imaging was performed for 24 hours, during which deionized water must be continually and directly pumped onto the lens to account for evaporation of water from between the objective and glass bottom dish. This was accomplished by mounting a metal collar (i.e., the cap from the Leica Water Immersion Micro Dispenser) over the objective, and attaching it to a 10-mL syringe using flexible polymer tubing (Tygon). The syringe was placed in a syringe pump (New Era Pump Systems Inc., Model# NE-300), which was set to dispense 40 uL/hr.

Inhibitor treatment

For inhibition of Fgf signaling, embryos maintained in PTU were treated with 12 μm-24 μm (as indicated in each experiment) FGFR inhibitor SU5402 (Sigma-Aldrich, #SML0043) with 0.3% dimethyl sulfoxide (DMSO) or 0.3% DMSO for controls. Treatment was performed between 24 hpf and 48 hpf. Embryos were washed for five minutes in PTU before fixation with 4% PFA. To inhibit Wnt signaling, *Tg(hsp70:dkk1-GFP)* embryos were heat shocked at 24 hours post fertilization (hpf) according to published methods (Stoick-Cooper et al., 2007).

RESULTS

Progression of dHb development from *dbx1b*$^{+}$ progenitors to *cxcr4b*$^{+}$ precursors

Previous work proposed that transcripts for *dbx1b* and *cxcr4b* both are found in the progenitors of the dHb (Dean et al., 2014 Roussigné et al., 2009 Halluin et al., 2016). Expression of these two genes partially co-localize at 35 hours post fertilization (hpf) and 3 days post fertilization (dpf) (Fig. 13). The partial co-localization of these two expression domains suggest that these two genes may label different populations within the developing dHb.

The identity of the *dbx1b*$^{+}$ cells as progenitors of the dHb is well supported based on evidence from the fish (Dean et al., 2014) and from the *dbx1* mouse homolog (Vue et al., 2007). The *dbx1b*$^{+}$ progenitors give rise to neurons throughout the dHb and are located ventral and medial to dHb neurons in the larval zebrafish (Dean et al., 2014). We have confirmed these results, and extended the lineage tracing to adults (Fig. 14), where descendants of *dbx1b*$^{+}$ progenitors (mCherry^{+} nuclei) are found throughout neurons of the adult dHb, as visualized by *Tg(gng8:CAAX-GFP),* which labels dHb neurons and their efferent axons with membrane tagged GFP (deCarvalho et al., 2013). The exact identity of the *cxcr4b*$^{+}$ cells has not been described, and the relationship between the *cxcr4b* and *dbx1b*- expressing cells is poorly understood.

To characterize the behavior of *dbx1b*$^{+}$ and *cxcr4b*$^{+}$ cells in real-time, we used *TgBAC(dbx1b:EGFP)* and *TgBAC(cxcr4b:nls-tdTomato)* lines. Due to the known role of Cxcr4b in cell migration (refer to Lewellis & Knaut, 2012), we performed time-lapse imaging to determine whether *cxcr4b*$^{+}$ cells migrate to the developing dHb or whether *cxcr4b* expression is initiated *de novo* in *dbx1b*$^{+}$ progenitors. Images of doubly labeled

Fig. 13: Partial overlap in *cxcr4b* and *dbx1b* expression.

Visualization of *dbx1b* and *cxcr4b* transcripts by (A,B) colormetric *in situ* hybridization at 35 hpf or (C,D) fluorescent *in situ* hybridization at 3 dpf. (A,C) Dorsal (B) lateral and (D) frontal views show partial co-localization of expression in a subset of dHb cells (arrowheads). Scale bar, 30 μm.

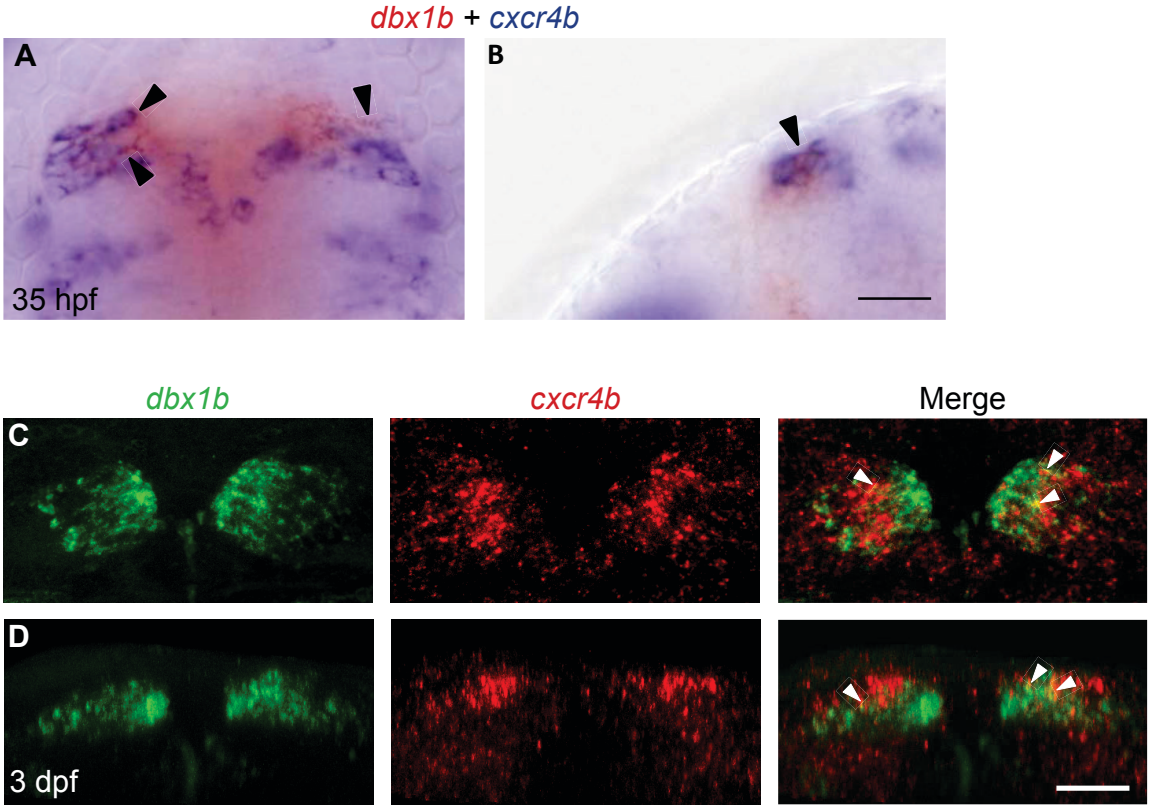
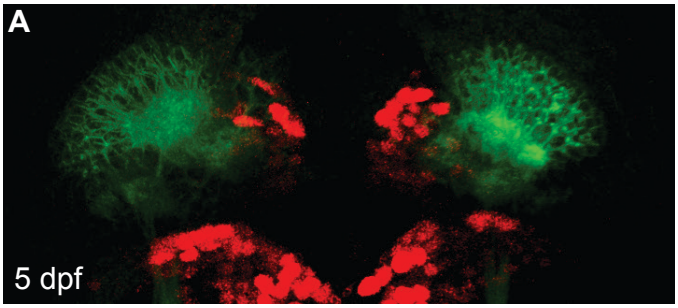

dbx1b + cxcr4b
A
B
35 hpf
dbx1b
cxcr4b
Merge
C
D
3 dpf

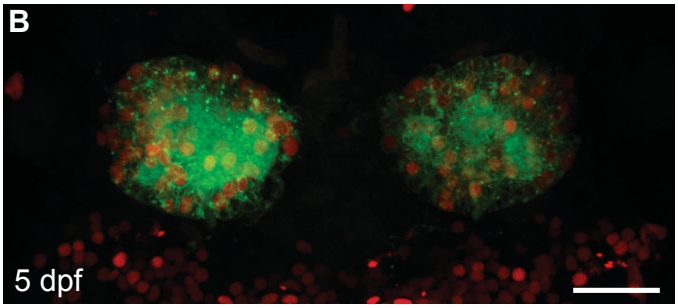
Fig. 14: Medial *dbx1b*$^{+}$ cells give rise to dHb neurons

TgBAC(gng8:CAAX-GFP) labels the cell membranes of dHb neurons (green). (A) *TgBAC(dbx1b:nls-dTomato)* labels cells medial and ventral to GFP labeled neurons. Dorsal view, 5 dpf. (B,C) Lineage tracing using Cre recombinase under control of a *dbx1b* driver [*TgBAC(dbx1b:Cre-mCherry)*] with a floxed reporter line [*Tg(ß-actin:loxP-hmgb1-eCFP-loxP-H2B-mCherry)*] labels neurons throughout the dHb with nuclear mCherry at (B) 5 dpf and (C) in adult brain sections. Scale bar (A,B) 30 μm and (C) 50 μm.

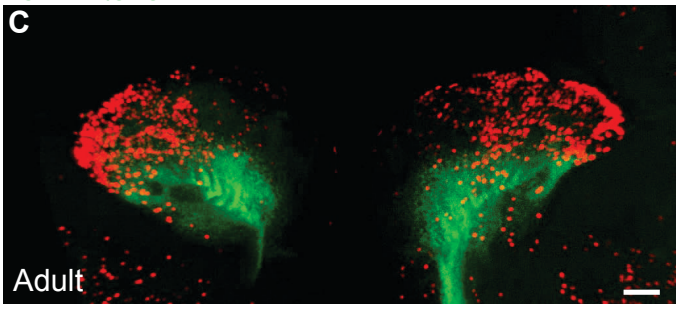
TgBAC(dbx1b:nls-dTomato);
TgBAC(gng8:GFP-CaaX



dbx1b Cre lineage tracing;
TgBAC(gng8:GFP-CaaX



dbx1b Cre lineage tracing;
TgBAC(gng8:GFP-CaaX



embryos were captured every 15 min between 27 and 48 hpf. At 27 hpf, GFP labeled dHb progenitors are detected in wild-type (WT) embryos and, by 32 hpf, dTomato^{+} nuclei appear within these GFP labeled cells (Fig. 15). In *wls* mutants, which produce fewer *dbx1b*$^{+}$ cells and have smaller dHb, there is a delay in the appearance of GFP and dTomato labeled cells (35 and 40 hpf, respectively, Fig. 15) and there are far fewer cells, consistent with a reduction in dHb progenitors. The restriction of dTomato nuclei to cells labeled by GFP in WT and *wls* mutants demonstrates that *cxcr4b* expression initiates within pre-existing *dbx1b*$^{+}$ progenitors. We found no evidence for migration of dTomato labeled cells into the presumptive habenular region.

At 2 dpf, dTomato-labeled nuclei are detected within the anterior dorsal region of the bilateral GFP labeled habenular domains (Fig. 16). By 3 dpf, GFP labeling becomes confined to a medial and ventral region of the developing dHb. The population of cells with dTomato labeled nuclei increases in the dorsal and lateral regions where GFP labeling is absent. However, a small number of co-labeled cells are found at the border between the GFP and dTomato uniquely labeled domains. Restriction of GFP to an increasingly smaller ventromedial region and the dorsolateral expansion of the dTomato labeled cell population continues over the course of several weeks.

Due to perdurance of dTomato labeling (Fig. 17), we also examined the distribution of endogenous *cxcr4b* transcripts by fluorescent RNA *in situ* hybridization. Double labeling for *ELAV like neuron-specific RNA binding protein 3* (*elavl3,* formerly known as *HuC*; Fig. 18A) or *anoctamin-2* (*ano2*; Fig. 18B), genes transcribed in mature dHb neurons (Kim et al., 1996; deCarvalho et al., 2014), indicates that *cxcr4b* expression is rapidly terminated as dHb neurons differentiate, though chemokine signaling persists in

dHb neurons (Fig. 25). These results suggest that *dbx1b*$^{+}$ progenitors undergo a transition to *cxcr4b*$^{+}$ neural precursors, which then give rise to differentiated neurons.

Fig. 15: Live imaging of *dbx1b*$^{+}$ and *cxcr4b*$^{+}$ labeled populations.

Live imaging stills captured between 27 and 48 hpf of WT and *wls* mutant *TgBAC(dbx1b:EGFP)* and *TgBAC(cxcr4b:nls-dTomato)* doubly labeled embryos. Corresponding images (black & white) of only the nuclear dTomato labeled population are shown. By 48 hpf, dTomato labeling is detected in GFP^{+} cells of the developing dHb (arrowheads) and the presumptive pineal (dashed circle) in WT and *wls* mutant embryos. Scale bar, 30 μm and 11 μm for inset.

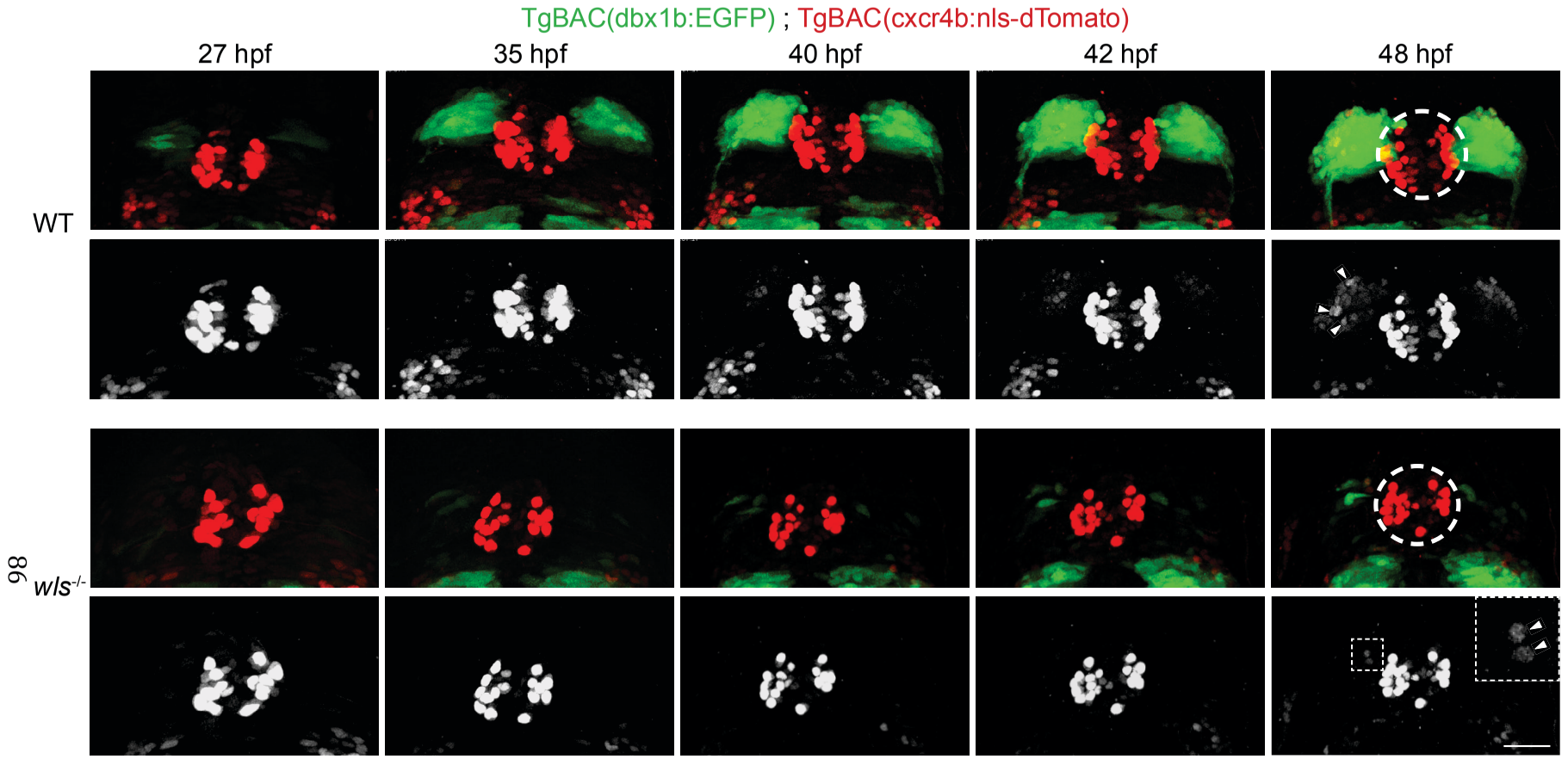

TgBAC(dbx1b:EGFP) ; TgBAC(cxcr4b:nls-dTomato)
27 hpf
35 hpf
40 hpf
42 hpf
48 hpf
WT
wls-/-

Fig. 16: Position of *dbx1b*$^{+}$ and *cxcr4b*$^{+}$ cells in the developing dHb.

(A) Relative positions of *dbx1b*$^{+}$ and *cxcr4b*$^{+}$ populations labeled with *TgBAC(dbx1b:EGFP)* and *TgBAC(cxcr4b:nls-dTomato)*, respectively, between 2 and 30 dpf. Scale bar, 30 μm.

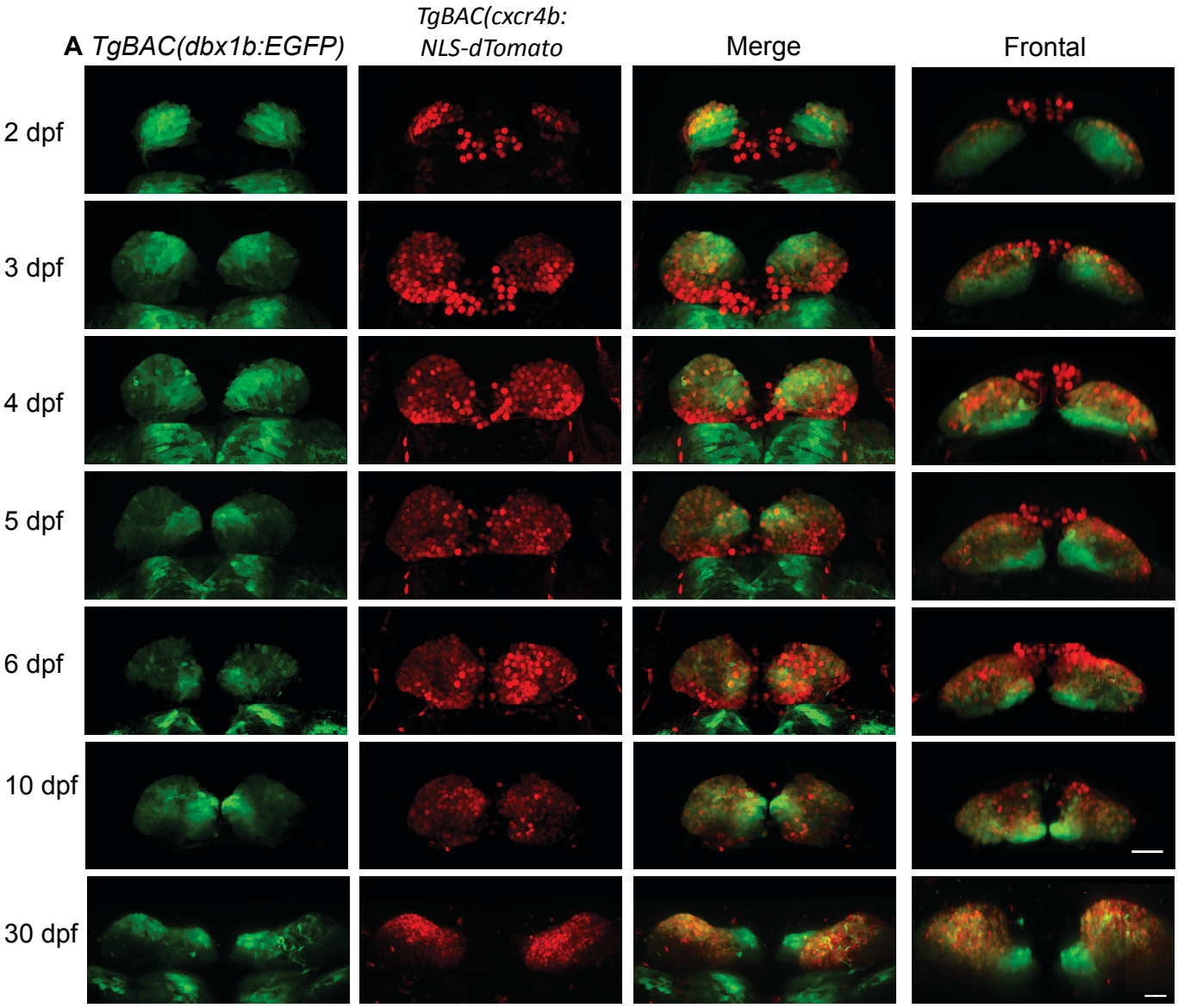

A TgBAC(dbx1b:EGFP)
TgBAC(cxcr4b:
NLS-dTomato
Merge
Frontal
2 dpf
3 dpf
4 dpf
5 dpf
6 dpf
10 dpf
30 dpf

Fig. 17: Persistence of fluorescent labeling in dHb neurons of transgenic larvae. In 5 dpf *TgBAC(cxcr4b:nls-tdTomato)* larvae, dTomato labeling persists in dHb neurons co-labeled with (A) *TgBAC(gng8:CAAX-GFP)* or (B) *Tg(HuC:H2B-GCaMP6s)*. Scale bar, 30 μm.

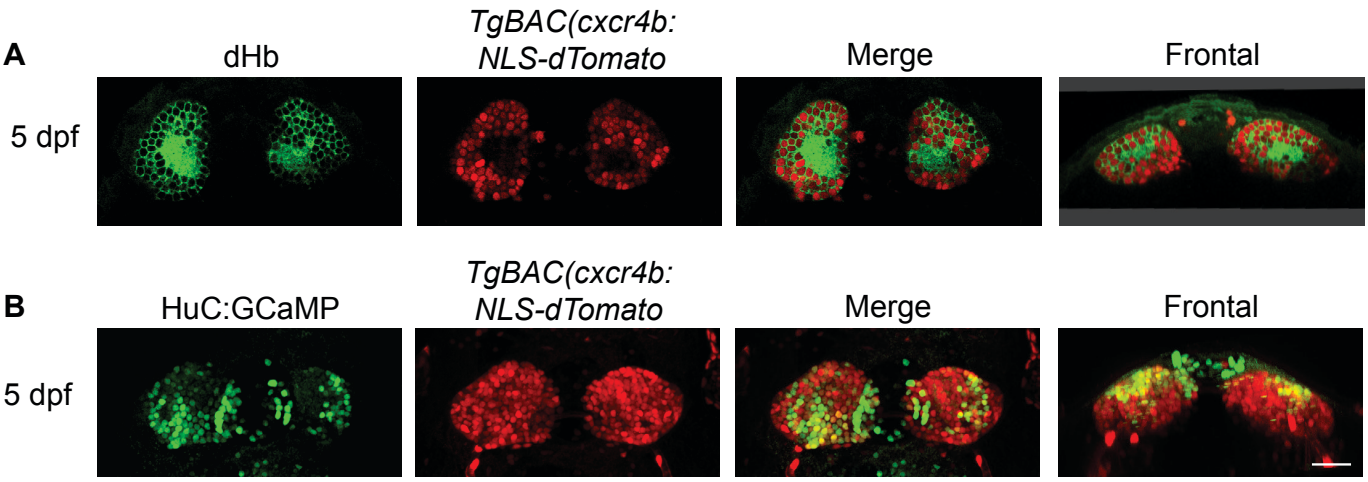
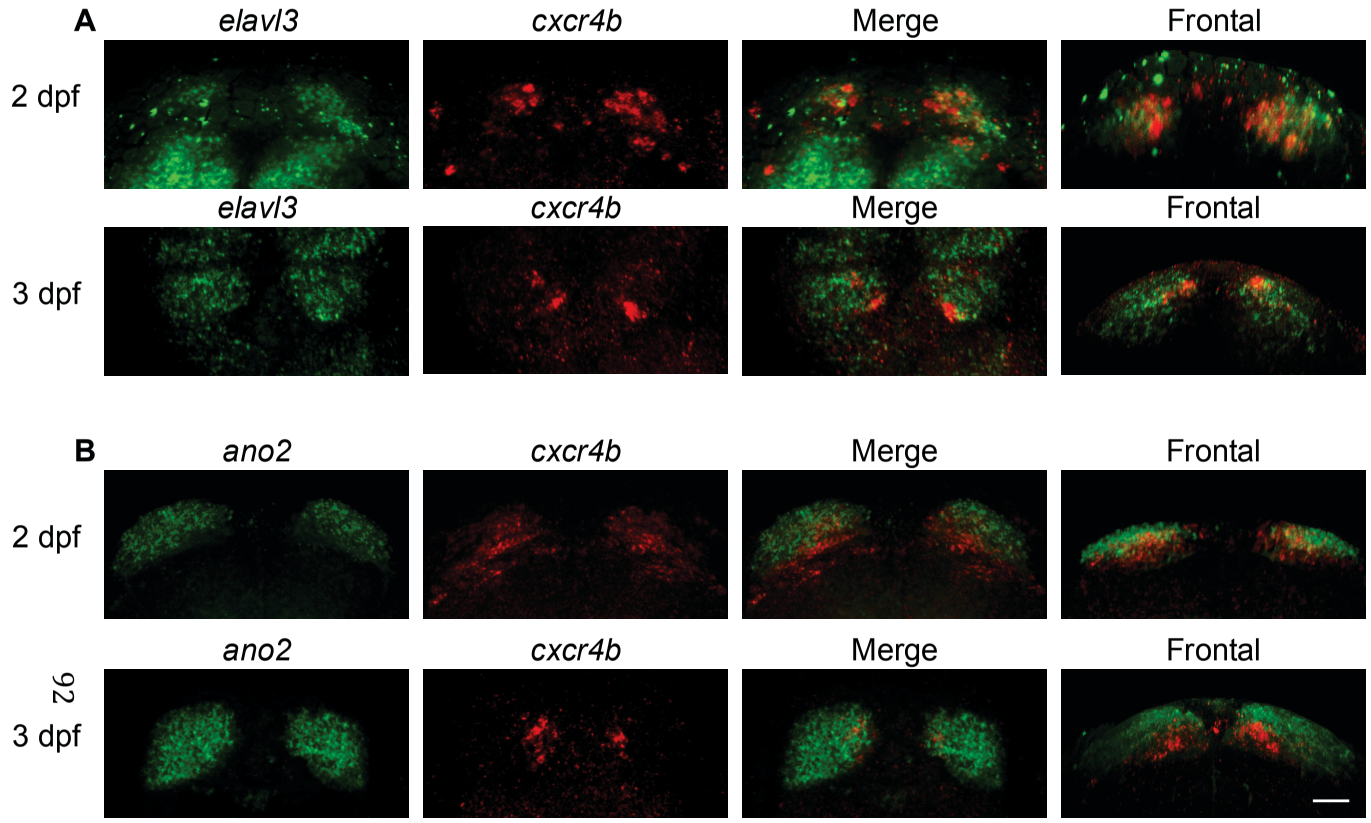

A
dHb
TgBAC(cxcr4b:
NLS-dTomato
Merge
Frontal
5 dpf
B
HuC:GCaMP
TgBAC(cxcr4b:
NLS-dTomato
Merge
Frontal
5 dpf

Fig. 18: Transcripts of *cxcr4b* are found ventral and medial to dHb neurons.

Fluorescence *in situ* hybridization for transcripts of *cxcr4b* and (A) neuronal marker *elavl3* or (B) dHb neuronal marker *ano2* in 2 and 3 dpf larvae. Left three panels are corresponding dorsal views. Right panels are frontal views. Scale bars 30 μm.

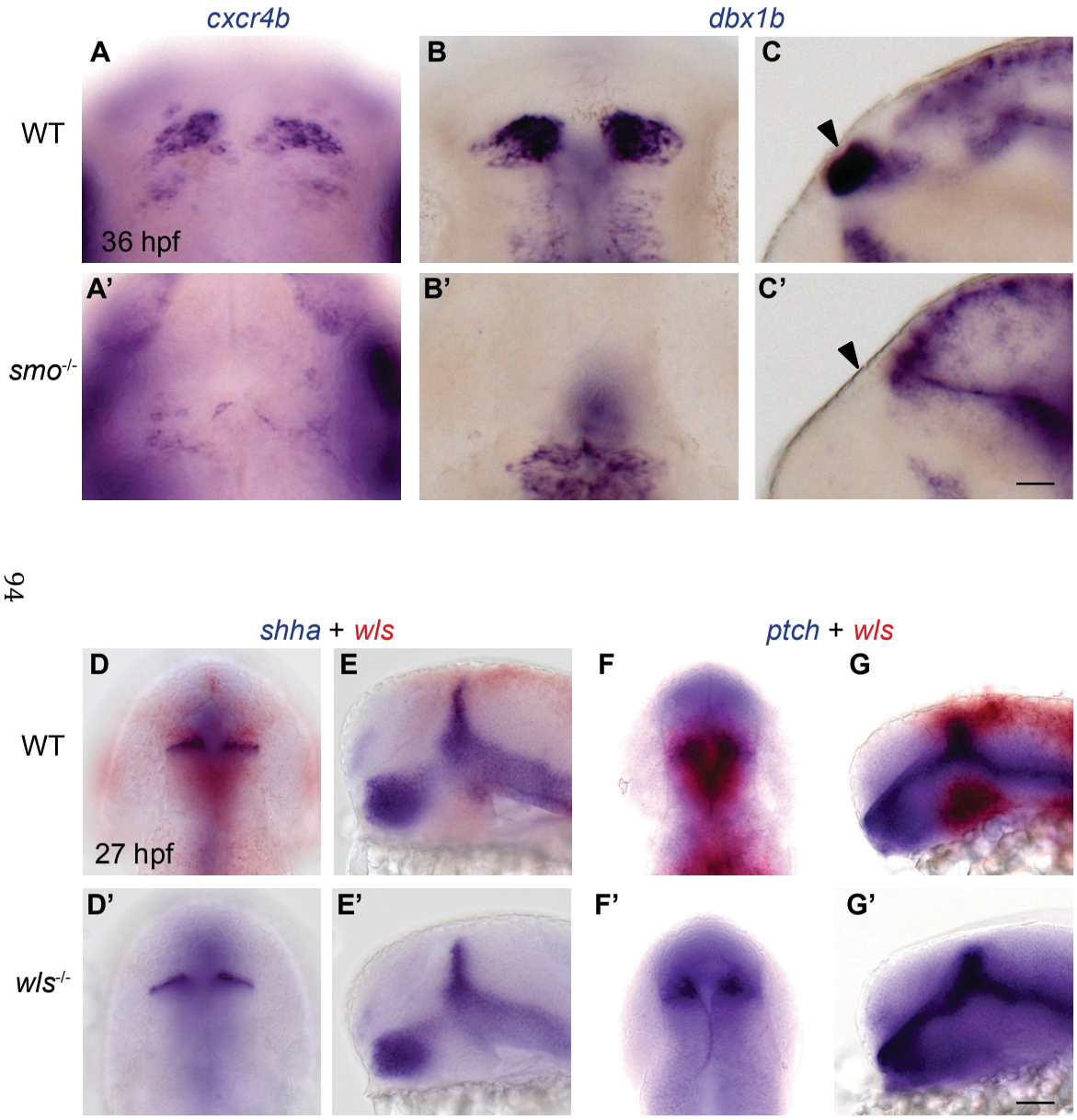

A
elavl3
cxcr4b
Merge
Frontal
2 dpf
elavl3
cxcr4b
Merge
Frontal
3 dpf
B
ano2
cxcr4b
Merge
Frontal
2 dpf
ano2
cxcr4b
Merge
Frontal
3 dpf

Hedgehog signaling acts upstream or independent of Wnt signaling to specify dHb progenitors

Previously, Sonic hedgehog (Shh) signaling from the zona limitans intrathalamica (ZLI) was found to be essential for formation of the dHb, as mutants for the Shh signaling component Smoothened (Smo) lack *cxcr4b* expression in this region of the brain (Halluin et al., 2016; & Fig. 19A,A'). We find that expression of *dbx1b* is also absent in *smo* homozygous mutants (Fig. 19B-C') indicative of the loss of dHb progenitors. To examine the relationship between Shh and Wnt signaling, we assessed expression of *sonic hedgehog a* (*shha*) and the Hh responsive gene *patched1* (*ptch1*) at the ZLI of *wls* mutants. Expression of both genes appeared indistinguishable from WT siblings (Fig. 19D-G'), suggesting that Shh signaling acts upstream or independent of Wnt signaling in the specification of dHb progenitors.

Fig. 19: Formation of *dbx1b*$^{+}$ dHb progenitors requires Shh signaling upstream of or parallel to Wnt signaling.

At 36 hpf, (A,A') *cxcr4b* and (B-C') *dbx1b* transcripts are found in the developing dHb of (A-C) WT embryos, but not in the corresponding brain region (A'-C') *smo* mutants. Expression of (D-E') *shha* and (F-G') *ptch1* is comparable in (D-G) WT embryos and their (D'-G') *wls* mutant siblings. Homozygous *wls* mutants were identified by their lack of *wls* transcripts (Kuan et al., 2015). (A,A',B,B',D,D',F,F') Dorsal views and (C,C',E,E',G,G') lateral views. Scale bar 30 μm.


cxcr4b
dbx1b
A
B
C
WT
36 hpf
A'
B'
C'
smo-/-
shha + wls
ptch + wls
D
E
F
G
WT
27 hpf
D'
E'
F'
G'
wls-/-

Diencephalic domain of Fgf signaling is restricted by Wnt signaling

Reduction of Fgf signaling using chemical inhibitors or in *fgf8a* homozygous mutants also results in fewer *dbx1b*-expressing progenitors (Dean et al., 2014) and small habenulae (Regan et al., 2009). Therefore, the reduced progenitor population observed in *wls* mutants could be due to a lack of Fgf signaling. In WT embryos, expression of *fgf8a* is limited to cells just anterior to the developing dHb (Fig. 20A-C). However, rather than being lost or smaller, the *fgf8a* domain is expanded posteriorly and medially in *wls* mutants (Fig. 20A'-C'). A similar enlargement of the *fgf8a* domain is seen after heat-shock induced over-expression of Wnt signaling inhibitor Dickkopf-1 (Dkk1) in 24 hpf *Tg(hsp70:dkk1-GFP*) embryos (Fig. 20D,D'). The result of expanded *fgf8a* expression is increased activity of Fgf signaling, as evidenced by a corresponding expansion in the expression domains of Fgf responsive genes such as *dusp6* and *etv5b* (Fig. 20E-H'). These findings indicate that, surprisingly, the reduction of dHb progenitors in *wls* mutants is not the result of decreased Fgf signaling. Instead, Wnt signaling normally acts to restrict the spatial extent of Fgf signaling in the dorsal diencephalon.

Given that Wnt and Fgf signaling are both important for the formation of *dbx1b*$^{+}$ dHb progenitors, we explored the epistatic relationship between these pathways. Transcripts for *dbx1b* are reduced in the dorsal diencephalon of *wls* and *fgf8a* single mutants compared to WT siblings. However, mutants doubly homozygous for *wls* and *fgf8a* completely lack *dbx1b* expression in this region and fail to form dHb (Fig. 20I-L and data not shown). In support of this finding, treatment of *wls* homozygous mutants with the Fgf receptor inhibitor SU5402, produces a similar phenotype, reducing *dbx1b*

expressing progenitors in a dose-dependent manner (Fig. 21A). Thus, the two signaling pathways act in an additive manner in the generation of dHb progenitors.

Fig. 20: Wnt signaling defines the domain of Fgf signaling in the dorsal diencephalon.

(A-C') At 35 hpf, the domain of *fgf8a* expression found rostral to the developing dHb is expanded in *wls* mutants. (D,D') Heat shock activation of the genetically encoded Wnt inhibitor *Tg(hsp70:dkk1-GFP)* at 36 hpf similarly expands the domain of *fgf8a* expression in 48 hpf embryos. Transcripts of (E,F) *dusp6* and (G,H) *etv5b*, two targets of Fgf signaling, are also expanded in (E',F',G',H') *wls* mutants. (I) Expression of *dbx1b* in the dorsal diencephalon is reduced in (J) *fgf8a* and (K) *wls* mutants, and absent in (L) *fgf8a;wls* double mutants. Dorsal views (A,C,,E,G) and lateral views (B,F,H,I-L) are provided. Absence of *wls* transcripts was used to confirm the *wls* mutant genotype. Scale bar is 50 μm.

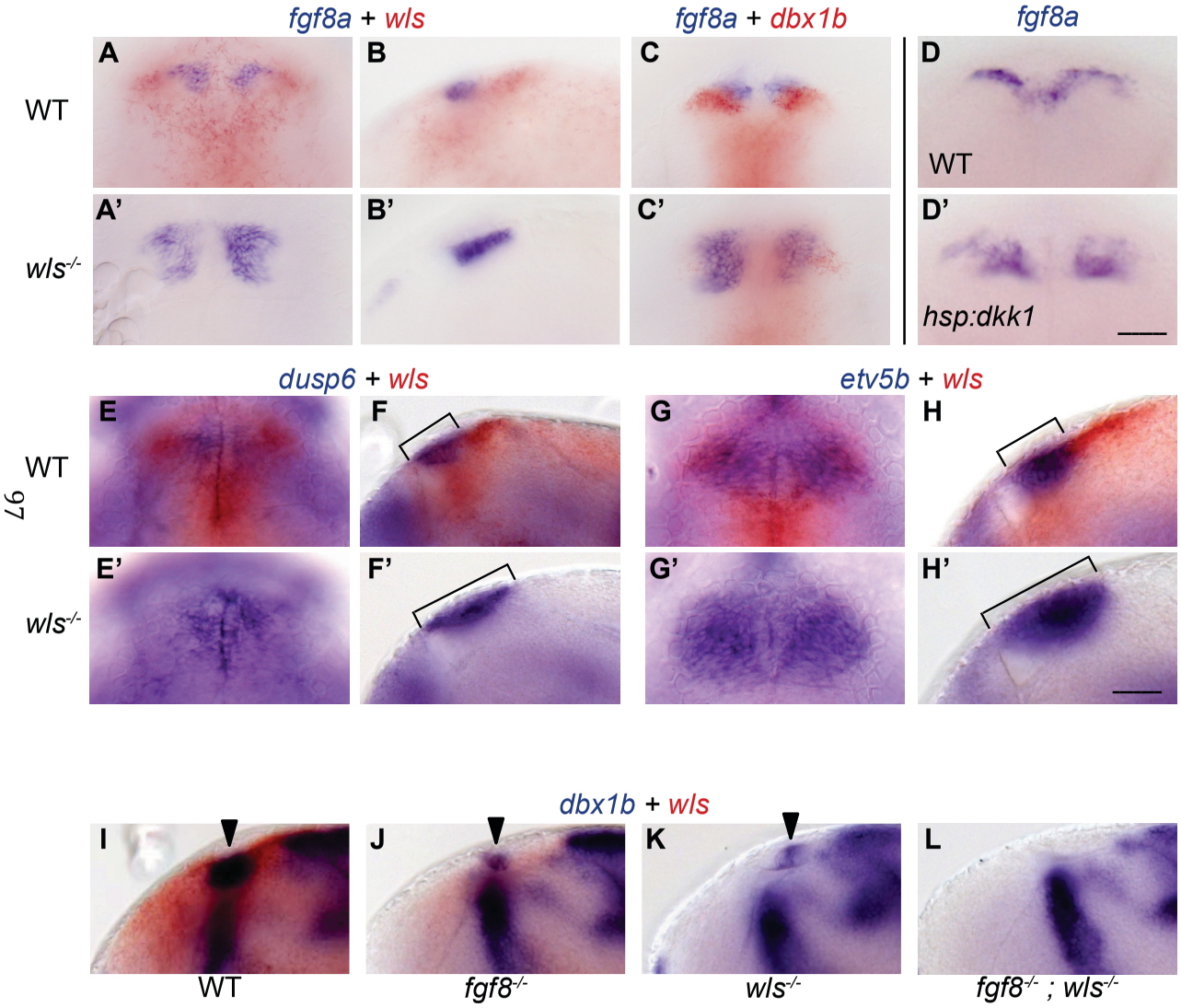
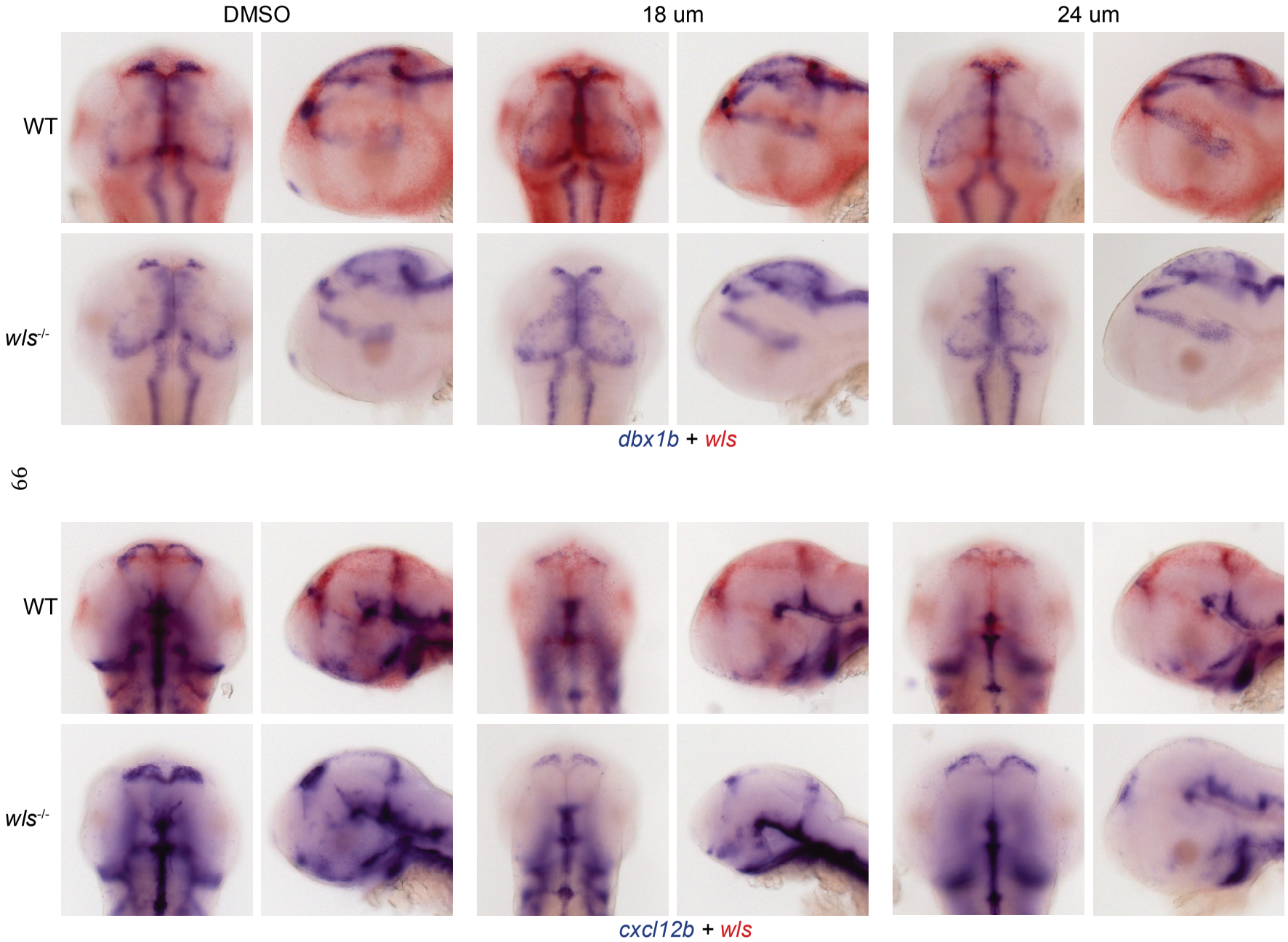

fgf8a + wls
fgf8a + dbx1b
fgf8a
WT
wls-/-
A
B
C
D
A'
B'
C'
D'
WT
hsp:dkk1
dusp6 + wls
etv5b + wls
WT
wls-/-
E
F
G
H
E'
F'
G'
H'
dbx1b + wls
I
J
K
L
WT
fgf8-/-
wls-/-
fgf8-/- ; wls-/-

Fig. 21: FGFR inhibitor reduces expression of *dbx1b* and *cxcl12b* in the diencephalic WT and in *wls* mutant embryos.

48 hpf embryos were treated for 24 hours with either 0.3% DMSO, 18 μM SU5402 + 0.3% DMSO, or 24 μM SU5402 + 0.3% DMSO, as indicated. (A) Diencephalic expression of *dbx1b* is reduced in a dose dependent manner in both WT and *wls* mutants, with no detectable levels of *dbx1b* in the developing dHb of *wls* mutants treated with the highest dosage. (B) The expression domain of *cxcl12b* is reduced in the diencephalon of WT embryos in a dose dependent manner. Conversely, the expression domain of *cxcl12b* is reduced in *wls* mutants, resulting in the restoration of the WT expression pattern at the highest dosage.


DMSO
18 um
24 um
WT
wls-/-
dbx1b + wls
WT
wls-/-
cxcl12b + wls

Opposing roles for Wnt and Fgf signaling in regulating the Crxc4b-chemokine pathway

In addition to formation of dHb progenitors, Wnt and Fgf signaling are necessary for proper expression of Cxcr4-chemokine pathway components. This pathway includes C-X-C motif ligand 12 chemokines (Cxcl12; formerly known as stromal cell-derived factor-1 or Sdf1), which bind to the Cxcr4 receptor and the atypical chemokine receptor 3 (Ackr3; formerly known as chemokine receptor 7 or Cxcr7). Cxcr4 is the signaling receptor, whereas Ackr3b sequesters excess Cxcl12 to establish a local gradient (Dambly-Chaudière et al., 2007; Lewellis & Knaut, 2012; Miyasaka et al., 2007; Boldajipour et al., 2008**).** Zebrafish have two Ackr3 and Cxcl12 homologues (a and b). At 35 hpf, *cxcl12a* transcripts are found in cells posterior to the *dbx1b*$^{+}$ developing habenulae (Fig. 22A-C), whereas *cxcl12b* is expressed in cells surrounding the habenular region, at higher levels anterior and lower levels posterior to it (Fig. 22D-F; Fig. 23A). Cells expressing *ackr3b* lie anterior to the *cxcl12b*$^{+}$ (Fig. 23B) and *dbx1b*$^{+}$ (Fig. 23C) populations, as well as in the midline between the developing dHb.

Disruption of Wnt or Fgf signaling significantly alters the expression patterns of *cxcl12b* and *ackr3b*. In *wls* mutants, *cxcl12b* is expressed ectopically within the developing habenulae and at high levels both anteriorly and posteriorly (Fig. 22G-H') and midline expression of *ackr3b* is expanded laterally and posteriorly (Fig. 22I-J'). Transcripts of *cxcl12a* transcripts appear unaffected in *wls* mutants (Fig. 23D).

The enlarged *cxcl12b* and *ackr3b* expression domains resemble the expanded *fgf8a* expression observed in *wls* mutants (Fig. 22G-J', Fig. 20A-C'), suggesting that Fgf8a positively regulates expression of these chemokine pathway members. Consistent

Fig. 22: Fgf signaling regulates expression of chemokine pathway components.
The (A-C) *cxcl12a* and (D-F) *cxcl12b* genes are transcribed in regions adjacent to the dHb at 35 hpf, as indicated by fluoresence *in situ* hybridization and immunolabeling for GFP in *TgBAC(dbx1b:EGFP)* embryos. The (G-H') *cxcl12b* and (I-J') *ackr3b* expression domains (brackets) are expanded in *wls* homozygotes. However, in *fgf8a* mutants (K-L') *cxcl12b* and (M-N') *ackr3b* dorsal diencephalic expression (arrowheads) are reduced. (O-R) As shown in G-H' and K-L', expression of *cxcl12b* is reduced in (P) *fgf8a* mutants and expanded in (Q) *wls* mutants. The (O) WT domain of *cxcl12b* expression is partially restored in (R) *fgf8a;wls* double mutants. (Dorsal views (A-F,G,G',I,I',K,K',M,M') and lateral views (H,H',J,J',L,L',N,N', O-R) Scale bar, 50 μm.

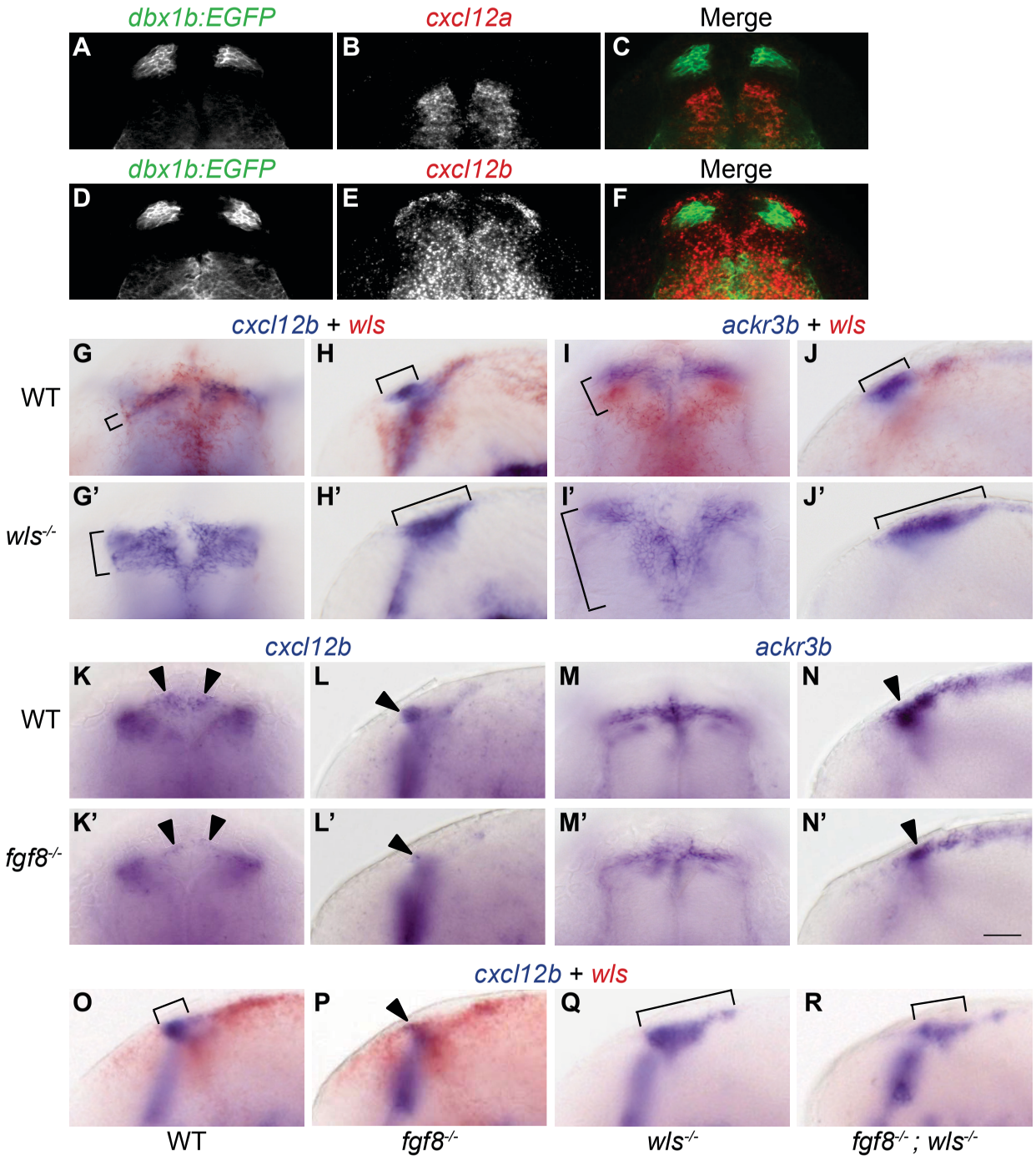
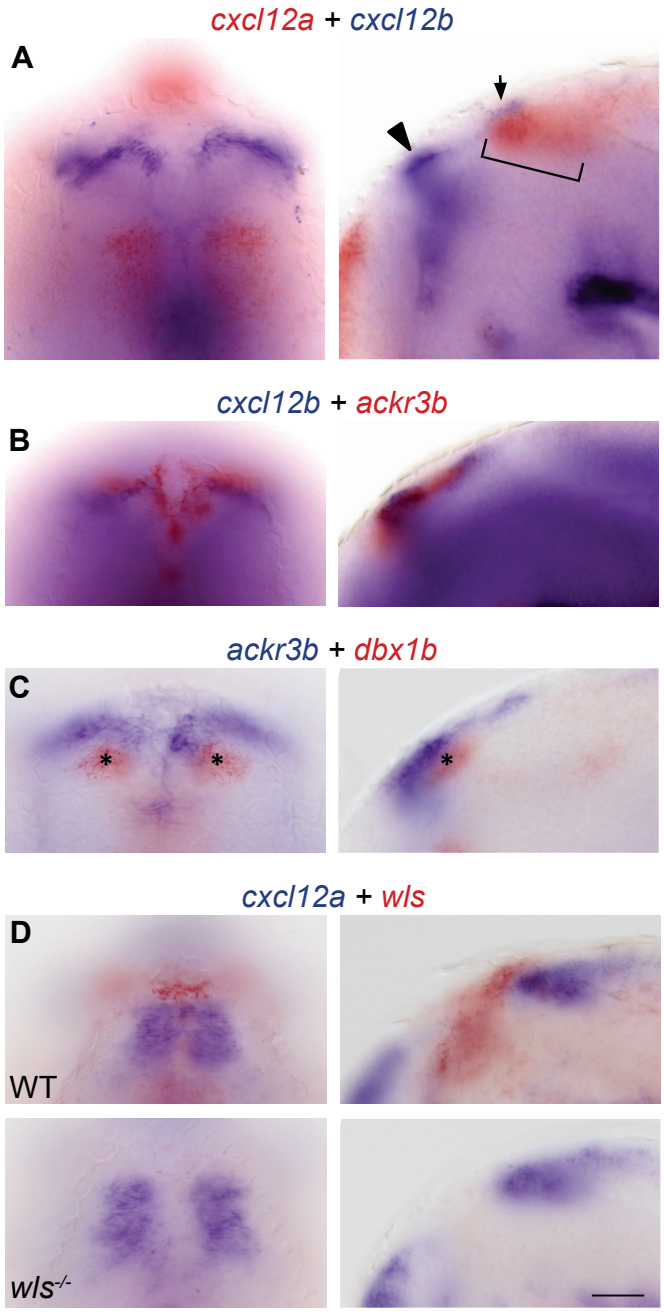

dbx1b:EGFP
cxcl12a
Merge
A
B
C
dbx1b:EGFP
cxcl12b
Merge
D
E
F
cxcl12b + wls
ackr3b + wls
WT
wls-/-
G
H
I
J
G'
H'
I'
J'
cxcl12b
ackr3b
WT
fgf8-/-
K
L
M
N
K'
L'
M'
N'
cxcl12b + wls
O
P
Q
R
WT
fgf8-/-
wls-/-
fgf8-/- ; wls-/-

Fig. 23: Diencephalic expression patterns of genes in the Cxcr4-chemokine signaling pathway.

(A) The *cxcl12a* gene is expressed caudal (bracket) to the developing habenulae, while *cxcl12b* transcripts are found in high levels rostral (arrowhead) and lower levels caudal (arrow) to the dHb. (B) Transcripts for *ackr3b* are found anterior and medial to the rostral domain of *cxcl12b* expression and (C) the *dbx1b*$^{+}$ progenitors (asterisks). (D) Expression of *cxcl12a* is not affected in *wls* mutants. Dorsal (left) and lateral (right) views. All embryos are 35 hpf. Scale bar, 50 μm.


cxcl12a + cxcl12b
A
cxcl12b + ackr3b
B
ackr3b + dbx1b
C
cxcl12a + wls
D
WT
wls-/-

with this, the *cxcl12b* domain in the dorsal diencephalon is reduced in *fgf8a* homozygous mutants at 35 hpf (Fig. 22K-L'). Expression of *ackr3b* is also affected but to a lesser degree (Fig. 22M-N'). Signaling by Wnts is epistatic to that mediated by Fgf8a, as in *wls;fgf8a* double mutants (Fig. 22O-R) or in *wls* mutants treated with SU5402 (Fig. 21B), *cxcl12b* expression is restored to a wild-type pattern. Together, these results indicate that Fgf and Wnt signaling act antagonistically to define the expression domain of chemokine signaling pathway members.

Cxcr4b chemokine pathway is necessary for directional outgrowth of dHb axons

The function of the Cxcr4b-chemokine pathway in the developing dHb is unknown. Chemokine signaling is known to mediate cell cohesion and migration (Miyasaka et al., 2007; Palevitch et al., 2010; Aman & Piotrowski, 2008); however, the size and morphology of the dHb appear normal in *cxcr4b* homozygous mutants (Fig. 24A,B; data not shown). Because Cxcr4-related receptors are also involved in axonal outgrowth and pathfinding by diverse neuronal types (Li et al., 2005; Miyasaka et al., 2007; Chalasani et al., 2003a; Chalasani et al., 2003b; Chalasani et al., 2007; Lieberam et al., 2005), we examined the projections of dHb neurons.

As visualized by *Tg(gng:CAAX-GFP)* labeling (Fig. 24A), axons of dHb neurons typically emerge at a lateral posterior position and fasciculate in the prominent bilateral fiber bundles of the fasciculus retroflexus (FR) that extend posteriorly to the midbrain IPN. We found that the majority of *cxcr4b* mutants have defective dHb axonal projections (Fig. 24B,D). Axonal phenotypes were categorized into three classes: In Class I larvae, the dHb-IPN projection is indistinguishable from WT; Class II larvae exhibit

both anteriorly projecting axons and properly directed posteriorly projecting axons; in Class III larvae, axons from the left and right dHb merge to form a thick bundle that extends anteriorly along the midline. Axon pathfinding is predominantly normal in larvae that are WT or heterozygous for the *cxcr4b* mutation (85.4%; n= 41 larvae). Most homozygous *cxcr4b* mutants display Class II (48.6%) and III phenotypes (45.7%; n= 35). The directionality of axonal outgrowth is disrupted in a similar manner in *wls* mutant larvae (Fig. 24C,D; Class II 58.1%, Class III 14%; n= 43), consistent with the observed misregulation of *cxcl12b* and *ackr3b* expression (Fig. 23G-J'). Therefore, the Cxcr4b-chemokine signaling pathway, while not required for the formation of dHb neurons, is necessary for the correct outgrowth of their efferent axons.

Correspondingly, chemokine signaling is active in dHb neurons and axons (Fig. 25). Chemokine signaling can be assayed using a transgenic reporter *TgBAC(cxcr4b:cxcr4b-mKate2-IRES-GFP-Caax)* (Lewellis et al., 2013). The presence of the Cxcr4b-mKate2 fusion protein at the GFP^+ membrane is indicative of active signaling. Interestingly, the mKate2 labeling is detected in the medial and ventral regions of the dHb where newly-born neurons arise. Although GFP^+ labeling in axons extends from the dHb toward the IPN (Fig. 25, asterisks), the overlapping mKate2 signal is restricted to the anterior portion of the efferent projections (Fig. 25, arrows). The localization of reporter labeling further supports a role for chemokine signaling in the initial outgrowth of axons from dHb neurons.

Fig. 24: Chemokine signaling directs outgrowth of dHb axons.

(A) *TgBAC(gng8:CAAX-GFP)* labels the left and right dHb, FR and IPN of WT larvae. Four phenotypic classes of dHb axonal morphology can be distinguished in (B) *cxcr4b* mutants and (C) *wls* mutants. The dHb (asterisks) and defasciculated axons (arrowheads) are indicated. Scale bar, 50 μm. (D) Percentage of WT siblings (blue, n= 41), and *cxcr4b* (red, n= 35) and *wls* (green, n= 43) mutants within Classes I-III.

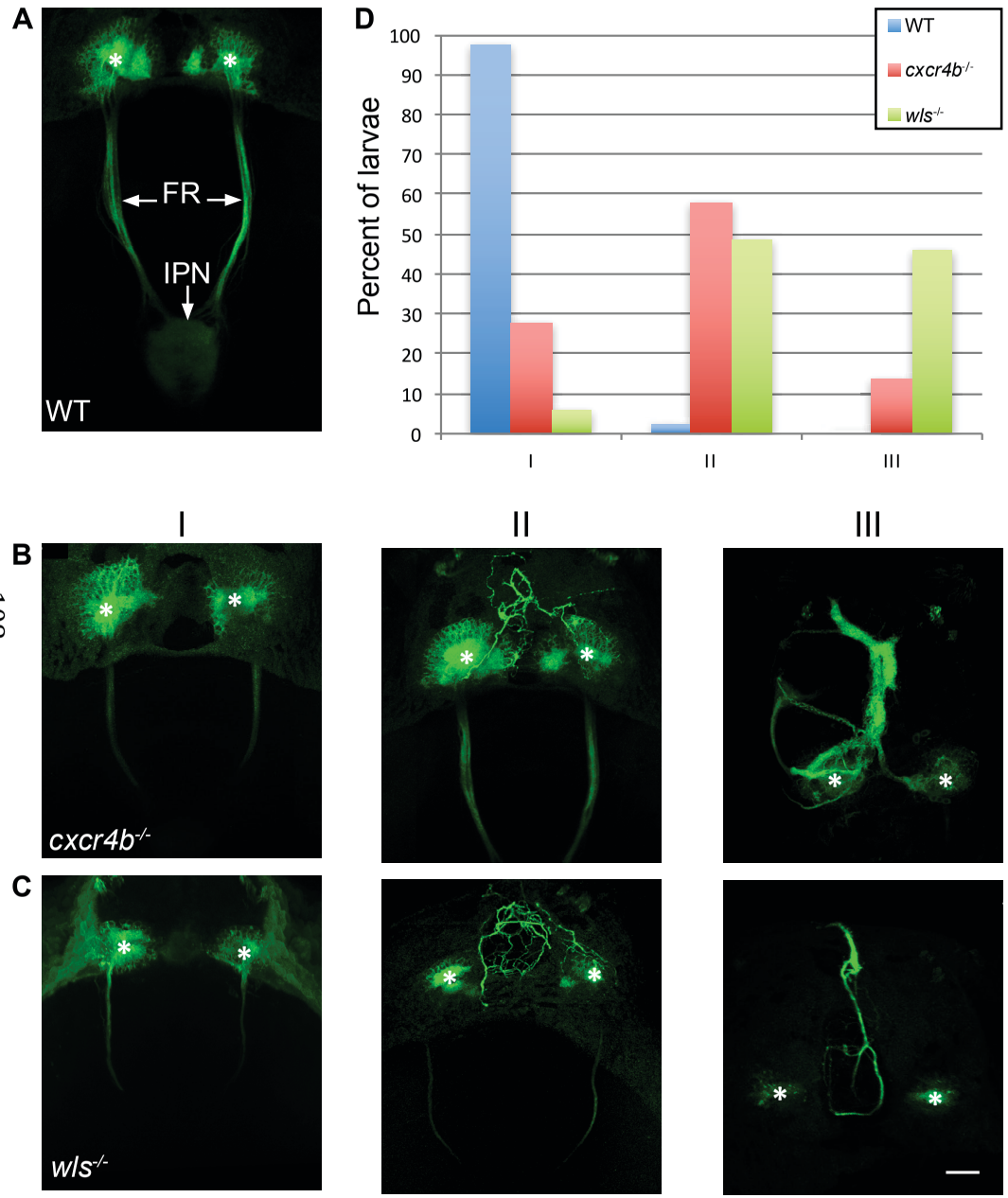
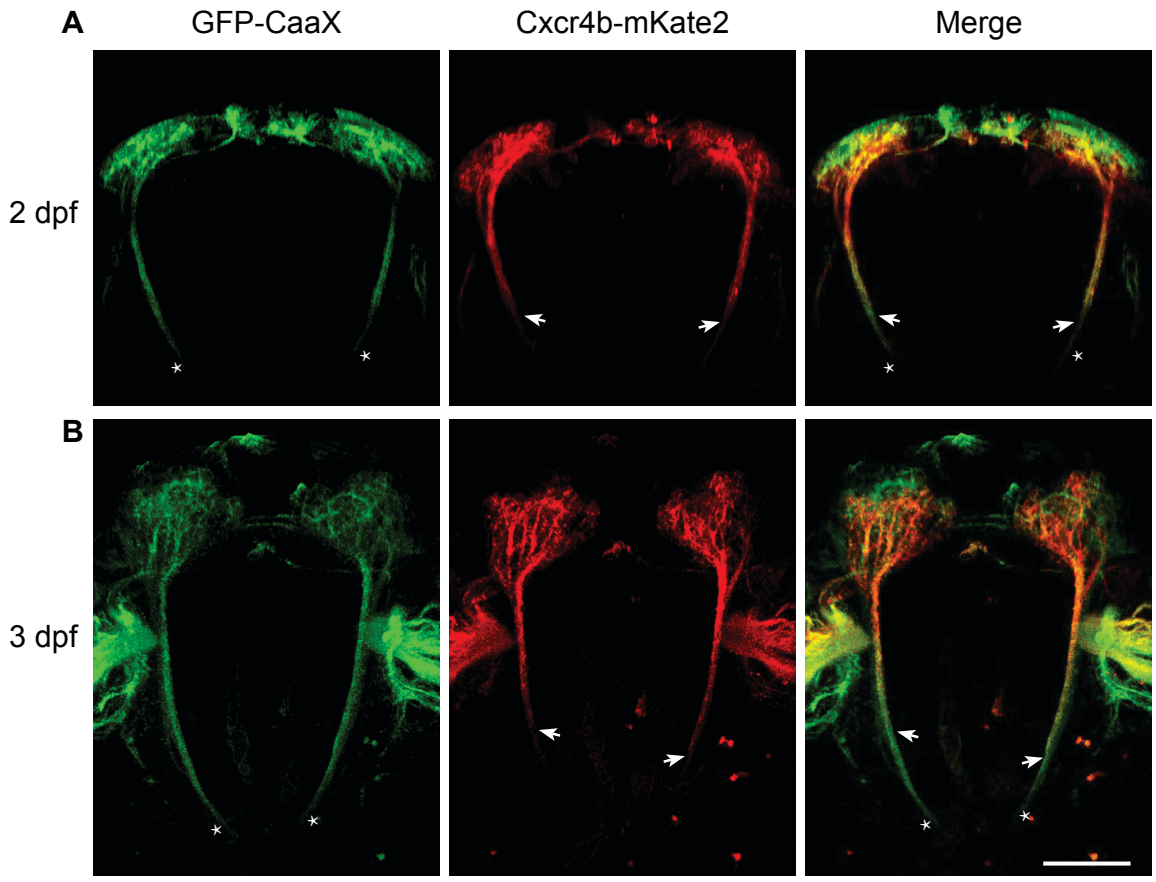

A
FR
IPN
WT
D
Percent of larvae
100
90
80
70
60
50
40
30
20
10
0
WT
cxcr4b-/-
wls-/-
I
II
III
B
cxcr4b-/-
C
wls-/-

Fig. 25: Chemokine signaling is active in newly born neurons and their axons.

Labeling from *TgBAC(cxcr4b:cxcr4b-mKate2-IRES-GFP-CaaX)* at (A) 2 dpf and (B) 3 dpf. The membrane tagged GFP labels dHb neurons and axons, while actively signaling chemokine receptor is only seen at the membrane of neurons near the ventricular zone. Additionally, mKate2 labeling terminates more anterior in the FR (arrows) than labeling by GFP (asterisks). Scale bar, 50 μm.


A
GFP-CaaX
Cxcr4b-mKate2
Merge
2 dpf
B
3 dpf

DISCUSSION

We investigated the formation of the zebrafish dHb nuclei and examined how the convergence of signaling pathways influences their development. As summarized in Fig. 26, Shh acts upstream or parallel to Wnt signaling, which functions additively with Fgf signaling to generate *dbx1b*-expressing dHb progenitors. The progenitors then give rise to *cxcr4b*-expressing neural precursors that differentiate into the mature neurons of the dHb. A second role for Wnt signaling is to delimit the spatial extent of Fgf activity in the dorsal diencephalon, which, in turn, regulates the expression of *cxcl12b* and *ackr3b*. In the absence of Wnt signaling or upon loss of the Cxcr4b chemokine receptor, dHb neurons exhibit aberrant axonal outgrowth. Thus, we have established a sequence of developmental signals that direct progenitor formation, neuronal differentiation, and axon outgrowth.

Identity and differentiation of dHb progenitors

In prior studies, two populations of cells were presumed to correspond to habenular progenitors in zebrafish, cells expressing *cxcr4b* and those expressing *dbx1b* (Roussigné et al., 2009; Halluin et al., 2016; Dean et al., 2014). Because only a small subset of cells shows overlapping expression, we sought to determine the relationship between the cell populations that express these genes.

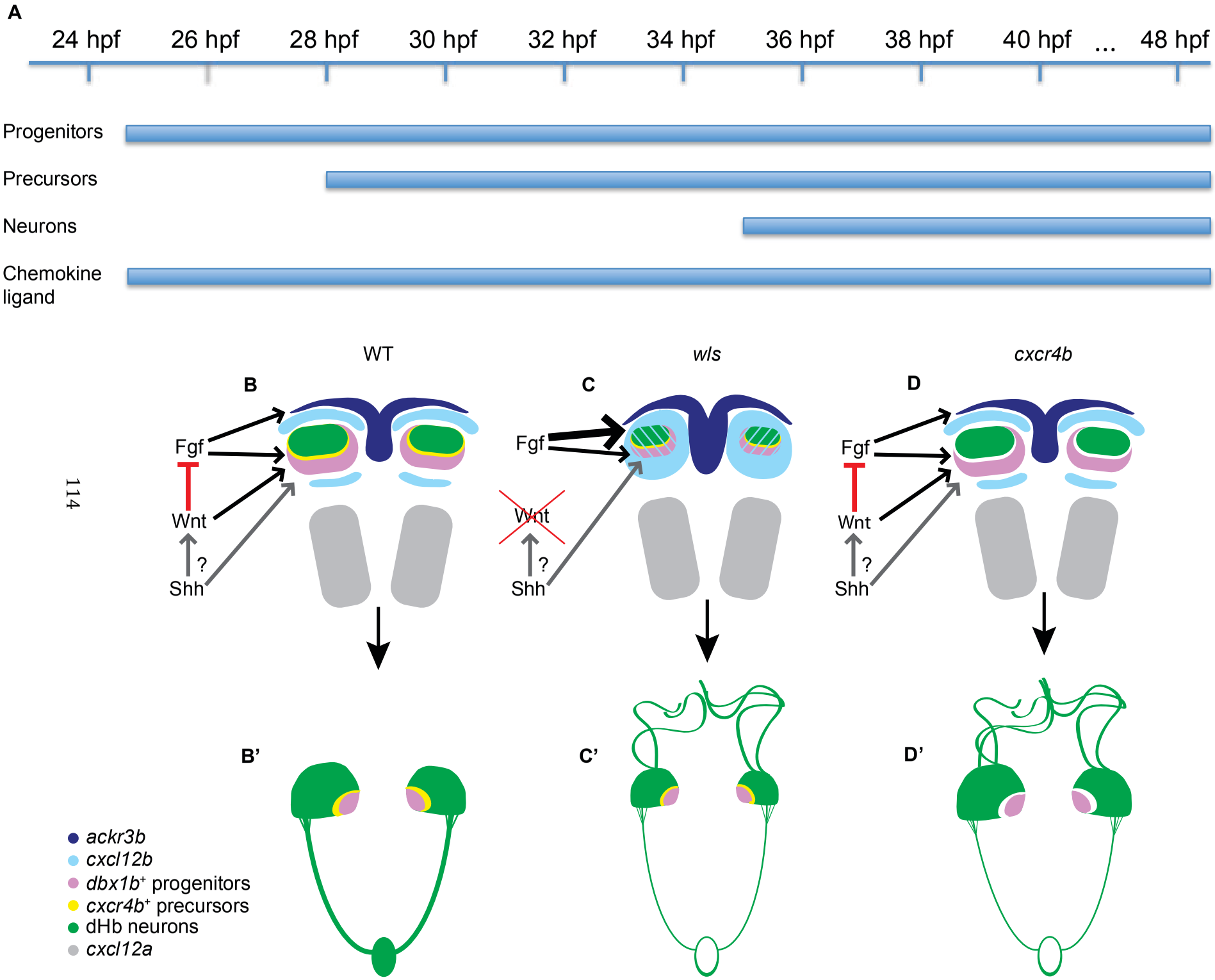
The earliest known marker of the developing dHb is *dbx1b,* with transcripts detected as early as 24 hpf (Dean et al., 2014). The *dbx1b* expressing cells are found in a ventromedial domain relative to mature habenular neurons. Further, our consecutive imaging of the dHb demonstrates that labeling from a *dbx1b* transgenic driver decreases

over time, becomes restricted to the most medioventral region, and persists until at least four weeks post fertilization. Lineage tracing experiments demonstrate that the *dbx1b*-expressing cells are progenitors as they give rise to neurons throughout the larval as well as the adult dHb. These findings are also in agreement with lineage tracing in the mouse brain and expression analyses with the murine homolog *dbx1* (Vue et al., 2007).

Expression of *cxcr4b* in the habenulae is first detected at 28 hpf, four hours later than *dbx1b* (Roussigné et al., 2009). Time-lapse imaging reveals that *cxcr4b* expressing cells do not migrate to the developing habenulae from more caudal regions, as had been previously proposed (Kuan et al., 2015). Rather, labeling from a *cxcr4b* driver is initiated in a subset of cells that express GFP under the control of a *dbx1b* promoter. This finding indicates that *cxcr4b*$^{+}$ cells are derived from the *dbx1b*$^{+}$ progenitors. Over time, *dbx1b* expression is downregulated and most *cxcr4b*$^{+}$ cells are found dorsal and lateral to the *dbx1b*$^{+}$ progenitors. Expression of *cxcr4b* is also gradually lost as cells in more dorsolateral positions begin to express markers of differentiated dHb neurons. The temporal pattern of *cxcr4b* expression parallels the previously described medial to lateral progression of neurogenesis (Aizawa et al., 2007). Perdurance of dTomato labeling from *TgBAC(cxcr4b:nls-dTomato)* in mature dHb neurons provides further support that *cxcr4b* expression demarcates a neural precursor population. Thus, development of the dHb is characterized by the transition from *dbx1b*$^{+}$ progenitors to *cxcr4b*$^{+}$ neural precursors and finally to *elavl3*$^{+}$ neurons.

Fig. 26: Integration of multiple signaling pathways in dHb development.

(A) Timeline indicating when *dbx1b*+ progenitors, *cxcr4b*+ precursors, and *ano2*+ neurons can be detected in the developing dHb. Activity of Shh, Wnt and Fgf signaling as well as expression of both chemokine ligands *cxcl12a* and *cxcl12b* are present in the developing dorsal diencephalon during all time points (not indicated). (B) In zebrafish embryos, Wnt and Fgf signaling function additively to generate *dbx1b*$^{+}$ progenitors (purple), that produce *cxcr4b*$^{+}$ neural precursors (yellow), which differentiate into habenular neurons (green). Shh signaling acts upstream of or parallel to these pathways. Wnt signaling also regulates the extent of Fgf signaling, which in turn regulates *cxcl12b* (light blue) and *ackr3b* (dark blue) expression in regions anterior to the developing dHb. *cxcl12a* (gray) transcripts are present in bilateral domains posterior and ventral to the habenular region. Establishing the spatial pattern of chemokine signals later influences the outgrowth of dHb efferents. (B') In WT larvae, anterior inhibition or posterior attraction directs outgrowth of dHb axons caudally towards the IPN. (C) In *wls* mutants, Wnt signaling is disrupted, *dbx1b*$^{+}$ progenitors are reduced, and the dHb are significantly smaller (Kuan et al., 2015). Additionally, Fgf signaling is expanded, as are *cxcl12b* and *ackr3b* domains of expression, resulting in (C') defective chemokine signaling and aberrant rostral projections from dHb neurons. (D) In *cxcr4b* mutant embryos, habenular development appears normal, but (D') dHb axon outgrowth is abnormal.


A
24 hpf
26 hpf
28 hpf
30 hpf
32 hpf
34 hpf
36 hpf
38 hpf
40 hpf
...
48 hpf
Progenitors
Precursors
Neurons
Chemokine ligand
WT
wls
cxcr4b
B
C
D
Fgf
Wnt
?
Shh
B'
C'
D'
ackr3b
cxcl12b
dbx1b+ progenitors
cxcr4b+ precursors
dHb neurons
cxcl12a

Formation of dbx1b^{+} dHb progenitors requires Wnt and Fgf signaling

Disruption of the Fgf or Wnt signaling pathways results in small dHb due to a reduction in progenitors (Regan et al., 2009; Dean et al., 2014, Kuan et al., 2015). It was previously reported that embryos homozygous for a mutation in *fgf8a* fail to form *dbx1b*-expressing progenitors (Dean et al., 2014). However, we find that *dbx1b* expressing cells are present in *fgf8a* homozygous mutants at a later stage, although there are considerably fewer in number than in WT siblings. Fgf signaling could be responsible for generating the appropriate number of progenitors and/or for regulating *dbx1b* transcription. In support of the latter, *dbx1b* expression was lost upon treatment of zebrafish embryos with an inhibitor of Fgf receptor 1, but restored after its removal (Dean et al., 2014)

Through the analysis of *wls* mutants, we discovered an unsuspected role for the Wnt pathway in spatially restricting expression of *fgf8a,* and thereby limiting the extent of Fgf signaling in the developing dorsal diencephalon. In *wls* mutants, however, increased Fgf signaling does not compensate for the loss of Wnt signaling, as there are still fewer *dbx1b*-expressing dHb progenitors. Furthermore, *wls; fgf8a* double mutants and *wls* mutants treated with an inhibitor of Fgf signaling fail to produce *dbx1b*$^{+}$ progenitors, indicating that the two signaling pathways work in an additive manner to generate the dHb progenitor population.

One hypothesis is that Wnt establishes a pro-habenular domain that will express *dbx1b* only after activation by Fgf signaling. Loss of Wnt signaling, as in *wls* mutants, results in smaller pro-habenulae and, therefore, fewer cells that could express *dbx1b*, irrespective of the level of Fgf signaling. Alternatively, both Wnt and Fgf signaling may contribute to the specification of the pro-habenulae. Distinguishing between these

hypotheses, and analyzing the progenitor population in the absence of *dbx1b* is challenging, since *dbx1b* expression is the earliest known indicator of dHb formation.

Recent studies have also implicated Hh signaling as an early regulator of habenular development (Chatterjee et al., 2014; Halluin et al., 2016). In zebrafish embryos homozygous for a mutation in the *smo* gene, which encodes a G protein-coupled like receptor essential for Hh signaling, expression of *cxcr4b* in the epithalamic region has both been reported as being absent (Halluin et al., 2016) and expanded (Chatterjee et al., 2014). We find a complete loss of *cxcr4b* as well as *dbx1b* expression in *smo* mutants, indicating that Hh signaling is required to produce dHb progenitors and/or to induce *dbx1b* expression. Based on these results, and the finding that expression of *shha* is unperturbed at the ZLI of *wls* homozygotes, the Hh pathway is expected to act upstream of or parallel to Wnt signaling in defining the pro-habenular territory.

Fgf and Wnt signaling regulates expression of chemokine pathway components

Our results demonstrate that Fgf8a normally functions downstream of Wnt signaling to define the spatial expression of chemokine pathway components in regions surrounding the habenulae. In *wls* mutants, *fgf8a* expression and Fgf signaling domains are dramatically enlarged, which is correlated with expansion of the *cxcl12b* and *ackr3b* expression domains. In contrast, homozygous *fgf8a* mutants show reduced expression of *cxcl12b* and *ackr3b*. Consistent with this, reduction of Fgf signaling through loss of Fgf8a or chemical inhibition of Fgf signaling results in a partial restoration of the WT *cxcl12b* expression domain.

A regulatory relationship between Wnt signaling, Fgf signaling and *ackr3b* expression was previously reported in the migrating lateral line primordia of zebrafish (Aman & Piotrowski, 2008). In this tissue, Fgf and Wnt signaling negatively interact to create mutually exclusive regions, where Wnt signaling is active at the leading tip of the migratory primordium and Fgf signaling occurs in the trailing zone (Aman & Piotrowski, 2008). Expression of *ackr3b* is spatially restricted to the trailing cells, either due to inhibition by Wnt in the leading zone, or activation by Fgf and/or relief of Wnt inhibition in the trailing cells. Although the details of the genetic interactions in the lateral line may differ from those in the dorsal diencephalon, the parallels between the two suggest a generally used developmental circuit that spatially confines the domain of chemokine signaling.

The Cxcr4-chemokine pathway directs habenular axon outgrowth

We have uncovered a new role for the Cxcr4-chemokine pathway in the establishment of dHb-IPN connectivity. Loss of the chemokine receptor Cxcr4b results in atypical rostral projections of habenular efferents. Similar results are seen in the *wls* mutant, in which expression of genes encoding chemokine ligand Cxcl12b and chemokine receptor Ackr3b are misregulated.

The function of Cxcl12 chemokines in axonal pathfinding is complex and depends on the neuronal type being assayed. For example, *cxcl12* is a repulsive cue for efferents of rat cerebellar neurons (Xiang et al., 2002), but an attractant for axons of retinal ganglion cells (Li et al., 2005), and creates a permissive environment for a number of cell types including olfactory neurons (Miyasaka et al., 2007) retinal ganglion cells

(Chalasani et al., 2003a, Chalasani et al., 2007), dorsal root ganglia neurons (Chalasani et al., 2003b), and ventral motor neurons (Lieberam et al., 2005). Thus, the projection defects of dHb neurons that result from loss of Cxcr4b could be due to the inability of their axons to respond to a posterior attractive cue and/or an anterior repulsive cue. The *cxcl12a* gene is expressed posterior to the developing habenulae and the chemokine it encodes could serve as an attractant that guides axons caudally. Conversely, *cxcl12b* transcripts are abundant anterior to the dHb, suggesting that Cxcl12b might act to repel axons from extending rostrally. Analyses of dHb axons in zebrafish larvae mutant for *cxcl12a* or *cxcl12b* will be required to distinguish their respective functions in regulating the direction of dHb axon outgrowth.

In *wls* mutants, expression of *cxcl12a* is unaltered and *cxcr4b* is expressed in the developing dHb, yet axons still project aberrantly. However, *cxcl12b* is inappropriately transcribed throughout the dHb. High levels of chemokine ligands have been reported to function as dominant negatives for chemokine signaling by driving internalization of the receptor (Miyasaka et al., 2007). It is possible that the expanded *cxcl12b* expression in *wls* mutants also has a dominant negative effect, resulting in rostral axonal projections. In *cxcr4b* and *wls* mutants, the majority of rostrally directed axons from both the left and right dHb extend towards the midline, where they fasciculate and project anteriorly as a single bundle (Class III phenotype). The dHb axons may therefore respond to a rostral anterior midline cue in the absence of normal chemokine signaling.

Axons that do emerge caudally from mutant dHb successfully join the FR and innervate the IPN. Thus, the chemokine signaling pathway is not required for guidance of dHb neurons to the target, but rather influences the initial directional outgrowth of their

axons. Newly born neurons and their emerging axons, but not older neurons or axons approaching or innervating the IPN, would therefore be expected to respond to chemokine signals. Indeed, chemokine signaling is restricted to the medial neurons, found closest to the ventricular zone. Active signaling is also detected in axons as they exit the dHb, but eventually is down-regulated as they extend closer to the IPN.

The successful pathfinding of some dHb axons also suggests that other signaling pathways mediate axon fasciculation, extension of the FR through the midbrain, and target recognition. Several axon guidance cues and receptors have been implicated in aspects of this process including Netrin/DCC (Schmidt et al., 2014; Funato et al., 2000), Sema3F/Nrp2 (Giger et al., 2000; Chen et al., 2000; Sahay et al., 2003), Sema5A (Kantor, 2004) and Sema3D/Nrp1a (Kuan et al., 2007). Disruption of most components leads to defasciculation of the FR or to incorrect innervation of the IPN. Only the loss of DCC or Netrin results in ectopic projections outside of the FR (Schmidt et al., 2014).

We have uncovered a complex network of signaling pathways that control the generation of dHb progenitors and their axonal outgrowth. Understanding the processes underlying development of the habenular nuclei and the generation of their neuronal diversity and connectivity is key to unraveling their roles in behavior and mood disorders. Here we provide a functional understanding of the relationships between the Wnt, Fgf, Shh and chemokine signaling pathways, which is an important step towards determining how dHb neurons arise and form appropriate synaptic connections.

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Chapter 4: Conclusion

In early 2013, President Obama announced the start of the Brain Research through Advancing Innovative Neurotechnologies (BRAIN) Initiative, intended to be an "all hands on deck" enterprise to uncover the basis for psychological illnesses and brain disorders, as well as develop new technologies to better understand brain function. Over $300 million has been put into this initiative, and it reflects the cooperative effort of the National Institutes for Health, Defense Advanced Research Projects Agency, Food and Drug Administration, National Science Foundation, Intelligence Advanced Research Projects Activity, and numerous private sector research institutions and companies (https://www.whitehouse.gov/share/brain-initiative). At the 2016 Ise-Shima G7 Summit, science academies around the world called for an international effort to establish a fundamental understanding of brain function and to identify the underlying etiologies of brain disorders, with the ultimate goal of developing new forms of disease management and treatment (Grillner et al., 2016).

To accomplish the goals proposed by these initiatives, researchers are generating whole-brain 3D maps of neurons and their connectivity using serial electron microscopy (Mikula & Denk, 2013), high resolution CT scanning (Cheng et al., 2011), and computational methods to overlay datasets onto brain atlases (Ullmann et al., 2010; Marquart et al., 2015; Marquart et al., 2016). In addition, functional data are added to neuroanatomical maps through directly investigating the contributions of discrete brain nuclei (Randlett et al., 2015). An important step towards the characterization and assessment of the roles of specific neural circuits is understanding how their neuronal subpopulations arise and form appropriate synaptic connections.

The focus of my research has been on the forebrain habenular nuclei, which comprise an important yet poorly understood integrating center, relaying information from the limbic system to regions throughout the brain (Namboodiri et al., 2016; Aizawa et al., 2011). The habenular nuclei are bilaterally positioned in the dorsal forebrain, and have been implicated in many clinically relevant psychological conditions, such as bipolar disorder, major depressive disorder, schizophrenia and addiction/withdrawal (Savitz et al., 2011; Ranft et al., 2010; Sartorius & Henn, 2007; Salas et al., 2009). In addition, the habenulae modulate basic cognitive states that are essential to survival such as fear/anxiety and reward, and behaviors such as sleep and mating (Okamoto et al., 2012; Jesuthasan, 2012; Yamaguchi et al., 2013; Facchin et al., 2009; Hikosaka, 2010; Aizawa et al., 2013; Huan et al., 1992; Sutherland, 1982; Modianos, 1974; Rodgers & Law, 1967).

In this thesis, I describe experiments that provide a more comprehensive picture of habenular development. Prior studies implicated the Shh and Fgf signaling pathways in regulating the generation of dHb progenitors, and Wnt signaling in establishing L-R asymmetry of the dHb. However, the involvement of other signaling pathways, how these signaling pathways interact with each other, and the relationship between two proposed progenitor populations, defined by *dbx1b* and *cxcr4b* expression, was unclear. To address these issues, I performed real-time imaging of the *cxcr4b*$^{+}$ and *dbx1b*$^{+}$ cell populations and clarified the developmental transition of dHb progenitors into mature neurons. The examination of *cxcr4b*-expressing cells led to the discovery of a new function for Cxcr4-chemokine signaling in guidance of dHb efferents. Additionally, the basis for the habenular defect of zebrafish mutant for the *wls* gene, which encodes a Wnt chaperone

protein, was determined. Notably, I integrated Shh, Wnt, Fgf, and chemokine signaling into a coherent pathway that regulates the formation of habenular progenitors and ultimately the outgrowth of axons from dHb neurons.

Signaling pathways in the development and connectivity of the dHb

Previously, dHb progenitors were defined by *dbx1* expression in mouse and *dbx1b* and *cxcr4b* expression in zebrafish (Vue et al., 2007; Quina et al., 2009; Dean et al., 2014; Roussigné et al., 2009; Halluin et al., 2016). Lineage tracing of *dbx1b*-expressing progenitors from zebrafish embryonic stages to adulthood show that they give rise to all dHb neurons. However, *dbx1b* and *cxcr4b* transcripts are found in adjacent domains in the developing dHb of zebrafish embryos, with co-expression in only a small subset of cells at their border (Dean et al., 2014). The majority of *cxcr4b* expression is found anterior and lateral to that of *dbx1b*-expressing cells, and ventral and medial to cells expressing markers of mature neurons. In addition, double labeling with transgenes under the control of a *cxcr4b* or *dbx1b* promoter shows that *cxcr4b* is transcribed in cells previously expressing *dbx1b.* From these results, I demonstrate that habenular development is characterized by a progression from *dbx1b*$^{+}$ progenitors to *cxcr4b*$^{+}$ neural precursors to mature dHb neuron.

Shh, Fgf and Wnt all have multiple and temporally distinct roles in the development of the forebrain, including the specification and regionalization of the diencephalon (Scholpp & Lumsden, 2010; Hagemann & Scholpp, 2012) and regulation of habenular development. Wnt signaling had previously been implicated in L-R asymmetry of the dHb and formation of the vHb (Beretta et al., 2013; Carl et al., 2007;

Hüsken et al., 2014). My work reveals an additional role for Wnt signaling in the generation of dHb progenitors, a function performed in conjunction with Fgf signaling and downstream of or parallel to Shh signaling. Intriguingly, the roles of Wnt signaling in habenular development are modular. For example, depending on the timing of signal disruption and the Wnt pathway gene that is altered, L-R asymmetry and dHb progenitor formation can be independently perturbed. The complex and reiterated use of the Shh, Wnt and Fgf signaling pathways leading to the generation of the dHb emphasizes the importance of knowing not only which signals influence the development of a structure, but also the precise developmental stages when they are active.

A signaling pathway may also be used repeatedly in different tissues at the same developmental stage. For example, Wnt signaling is required for progenitor formation, while concurrently regulating the spatial extent of *fgf8a* expression. Consequently, the domain of Fgf8a signaling dictates the region where genes encoding the chemokine components Cxcl12b and Ackr3b will be expressed. Although *cxcr4b* gene expression is frequently used as a marker for the developing dHb, the chemokine receptor it encodes is not essential for formation of the habenulae and its function was unknown. I discovered that the chemokine pathway has a role in directing posterior axon outgrowth from the dHb. In *wls* and *cxcr4b* mutants, a subset of axons project rostrally towards the telencephalon and frequently bundle at the midline anterior to the dHb. Additionally, chemokine signaling is active in newly born neurons and their projecting axons. Axon guidance cues, such as Semaphorin3F/5A and Netrin/DCC, that are known to influence axon pathfinding of dHb efferents are used by neurons in other brain regions. Therefore, there is a risk of dHb neurons responding to positionally inappropriate signals. I propose

that chemokine signaling provides a barrier, preventing dHb axonal outgrowth towards guidance cues located anteriorly.

Future directions

Although my studies have contributed to the understanding of the signaling pathways in dHb development and axon outgrowth, there are several unresolved questions for further avenues of exploration. One question is whether Dbx1b is required for habenular development. Overexpression of *dbx* (*Xdbx*) inhibits neural differentiation in Xenopus embryos, and thus, the Xdbx transcription factor is proposed to maintain neural progenitors (Gershon et al., 2000). However, in the mouse and fish, Dbx1 is required for correct D-V patterning of the spinal cord and generation of V0 interneurons (Pierani et al., 2001; Satou et al., 2012; Gribble et al., 2007). Loss of Dbx1a and Dbx1b in zebrafish results in a fate switch of V0 neurons to V1 neurons (Gribble et al., 2007). Transcripts of *dbx1b* are reportedly reduced in the developing dHb of *fgf8a* mutant zebrafish (Dean et al., 2014; Chapter 3), and these mutants form small dHb (Dean et al., 2014; Regan et al., 2009). These data suggest that the role of Dbx1b is to specify neural identity and that loss of Dbx1b leads to transfateing of neural progenitors in the spinal cord and possibly in the developing dHb. Generation of a zebrafish *dbx1b* mutant using CRISPR/Cas9 technology or examining the dHb of *Dbx1* mutant mice (Pierani et al., 2001) will address whether Dbx1b is required for dHb formation.

dbx1b is currently the only gene definitively shown to be transcribed in dHb progenitors. Therefore, we cannot determine if dHb progenitors are still present in mutants that lack *dbx1b* transcripts in the developing dHb. Other genes that label the dHb progenitor population will need to be defined and characterized. Two candidate genes,

wnt7b and *etv1*, have been preliminarily described in the mouse (Chatterjee et al., 2014). In the dorsal diencephalon of the mouse, *wnt7b* is co-expressed with *dbx1b*, though it is also expressed at high levels in the prethalamus. Expression of *etv1* is found between the *dbx1b*-expressing domains and the dHb neurons in mouse (Chatterjee et al., 2014) and in the habenulae of zebrafish (in zebrafish *etv1* was formerly known as *er81;* Roussigné et al., 2006). Lineage tracing shows that these *etv1*$^{+}$ cells do contribute to the medial habenulae, and likely label a precursor population (Chatterjee et al., 2014), though whether *etv1* is expressed in the same cells as those expressing *cxcr4b* remains to be seen. Further studies of *wnt7b* and *etv1* in this region will be needed to broaden our understanding of the molecular identity of dHb progenitors.

Shh, Wnt and Fgf signaling have all been implicated in the generation of the dHb (Chatterjee et al., 2014; Halluin et al., 2016; Carl et al., 2007; Hüsken et al., 2014; Regan et al., 2009; Dean et al., 2014). Shh and Wnt are hypothesized to regulate the specification of dHb progenitors, while Fgf signaling is believed to activate expression of *dbx1b* in this region. As outlined above, my findings suggest that Shh acts upstream or independent of Wnt signaling and that Wnt and Fgf work additively to generate the *dbx1b*-expressing dHb progenitors. However, it is unclear which Wnt proteins are involved and whether the three signaling pathways act directly or indirectly. For example, *dbx1b* promoter sequence needs to be analyzed for the presence of binding sites of Fgf signaling effectors. However, Fgf signaling is upstream of a variety of different effector proteins, many of which do not have characterized binding sequences. This will complicate the effort to determine whether Fgf signaling is directly upstream of *dbx1b* expression.

Future lines of research on the signaling pathways involved in dHb development may be informed by results from studies of the *dbx1b*$^{+}$ spinal cord progenitor populations. In the spinal cord, low levels of Shh signaling are believed to promote *dbx1* expression, while higher levels inhibit it (Wijgerde et al., 2002; Gribble et al., 2007). Other signaling pathways implicated include BMP and Wnt. BMP and Wnt signaling inhibit *dbx* expression to establish the dorsal boundary for expression of *dbx* genes in the spinal cord (Pierani et al., 1999; Novitch et al., 2003; Timmer et al., 2002; Gribble et al., 2007), although the exact mechanism for this inhibition has not been explored. Conversely, Wnt signaling is required for the generation of *dbx1b*-expressing dHb progenitors (Kuan et al., 2015; Chapter 2). However, to date, there is no evidence to implicate BMP signaling in the formation of dHb progenitors.

One important question is how a seemingly homogenous *dbx1b*$^{+}$ progenitor population generates a complex brain nucleus comprised of many different neuronal types. In the spinal cord, *dbx1*-expressing V0 progenitors give rise to glutamatergic, glycinergic and GABAergic neurons (Satou et al., 2012); a mixture similar to the dHb, which also is glutamatergic and GABAergic (deCarvalho et al., 2014). It has been hypothesized that the specification of multiple neuron types from *dbx1b*-expressing V0 progenitors is controlled through asymmetric cell division and temporal regulation (Satou et al., 2012). The temporal regulation of V0 neuron type reflects what is seen in the dHb, where Notch signaling regulates birth order and early-born dHb neurons adopt a different molecular identity than later-born neurons (Aizawa et al., 2007). These early born neurons predominantly contribute to the dHbL, while the later-born neurons predominantly contribute to the dHbM, and these dHb subdomains express different

genes and have different neuronal targets. However, how the birth order of dHb neurons influences the formation of these different neuronal identities will need to be explored. Additionally, how the timing of Notch signaling is regulated has not yet been determined.

If neural identity is temporally controlled, the matter of how long *dbx1b*$^{+}$ dHb progenitors are active must be addressed. The size of the habenulae continues to increase past larval stages, and GFP, under the control of a *dbx1b* driver, labels a presumptive progenitor population ventromedial to the dHb until at least 30 dpf. Most behavioral studies exploring the function of the dHb use larval or juvenile zebrafish. Therefore, this behavioral data may only represent the state of the nervous system at a given developmental stage as some neuronal types may have not yet formed, or neural connections by late-arising neurons have not yet been established. Analysis of dHb progenitor activity and proliferation at late stages of larval development and in juveniles will be required to determine the activity of progenitors late in development and whether the appearance new neuronal types correlates with increasingly sophisticated behavioral responses.

Newly formed neurons then send projections towards the IPN. My work reveals a new function for the Cxcr4-chemokine pathway in directing posterior axon outgrowth towards this midbrain target. As described earlier, the domains of expression of several genes in the chemokine pathway are regulated by interactions between Wnt and Fgf signaling; wherein Wnt signals restricts the expression of *fgf8a*, and therefore the domain of Fgf signaling, and together these signaling pathways demarcate the expression domain of chemokine pathway genes *cxcl12b* and *ackr3b*. According to the model, we expect to find transcription factor binding sites known to be responsive to Wnt signaling in the

promoter of *fgf8a*. The promoter sequences of *cxcl12b* and *ackr3b* should also be examined, though, for reasons addressed above, it will be difficult to identify Fgf signaling as a direct regulator. However, an alternative model has been proposed in the lateral line, where expression of *ackr3* is negatively controlled by Wnt signaling (Aman & Piotrowski, 2008). Compared to regulation by Fgf, the hypothesis that *ackr3b* is controlled by Wnt signaling will be more easily addressed.

My work reveals a new function for the Cxcr4b-chemokine pathway in habenular development. Abnormal chemokine signaling results in rostral axon projections. Interestingly, *cxcr4b* mutants, in which the chemokine receptor is lost, and *wls* mutants in which genes in the chemokine pathway are ectopically expressed at high levels, present a morphologically similar phenotype in perturbation of axon outgrowth. Overexpression of chemokine ligands can cause internalization of the chemokine receptor, and function as a dominant negative (Miyasaka et al., 2007). Consequently, it is possible that defects in axon outgrowth are due to loss of chemokine signaling in both *wls* and in *cxcr4b* mutants. Analysis of chemokine signaling in *wls* mutants is required to test this hypothesis.

An additional question is whether chemokine signaling repels anterior projections or acts to attract or create a permissive environment for posterior projections. Despite the aberrant outgrowth of dHb axons commonly observed in *wls* and *cxcr4b* mutants, some axons can respond to guidance molecules involved in fasciculation of the FR and innervation of the IPN. This is indicated by the successful pathfinding and innervation of the IPN by the subset of axons that do project posteriorly in mutant larvae. Furthermore, anterior projecting axons frequently fasciculate at the midline and project towards the telencephalon and olfactory bulb. This suggests that there is an anteriorly located cue to

which these axons can respond when chemokine signaling is disrupted. Therefore, the chemokine pathway may play a role in providing a stronger attractive cue in the posterior direction, or in blocking the response of dHb growth cones to anterior attractive cues. A third hypothesis is that inhibitory cues are present posterior to the dHb, and that Cxcl12a is required to create a permissive environment through which axons can project towards the IPN. Of the three hypotheses, this is the least likely, as *cxcr4b* mutants should have no chemokine signaling in the dHb, and yet many axons still successfully reach the IPN.

There are several ways to address the question of whether chemokine signaling acts in an anterior-repulsive or posterior-attractive manner. One method is by determining the directionality of chemokine signaling. Active chemokine signaling can be detected by the presence of a chemokine receptor at the cell membrane, while inactive receptor is localized to the cytoplasm. Using a transgenic approach, the amount of fluorescently-tagged Cxcr4b at the plasma membrane can be compared to the amount in the cytoplasm, allowing the presence of and directionality of Chemokine signaling to be determined (Venkiteswaran et al., 2013). Additionally, axonal trajectories of dHb neurons can be examined in *cxcl12a* and *cxcl12b* zebrafish mutants (Valentin et al., 2007; Bussmann et al., 2011) to determine whether chemokine signaling mediates anterior repulsion and/or posterior attraction. If chemokine signaling is attractive, loss of Cxcl12a posterior to the developing dHb should result in defective pathfinding to the IPN. Conversely, Cxcl12b is predominantly expressed anterior to the dHb, so if chemokine signaling normally repels these axons, then loss of Cxcl12b will result in ectopic anterior projections. If both signals play individual roles, then defects will be in *cxcl12a* and *cxcl12b* single mutants.

If anterior and posterior signals are mutually required, then axon outgrowth defects will be observed only in doubly homozygous *cxcl12a;cxcl12b* mutants.

A question not addressed in this study is whether inappropriate outgrowth of dHb axons affects neural survival, as neurons that fail to form connections with targets should die. At 5 dpf, the dHb of *wls* mutants are dramatically smaller than those of their WT siblings, though this defect is likely due to the reduced generation of dHb progenitors (Kuan et al., 2015; Chapter 2). In contrast, the dHb of *cxcr4b* mutants appear morphologically normal. However, analyses of dHb size and morphology has not been assessed at later time points. If size and morphology are unaffected in *cxcr4b* mutants, the aberrant dHb axons may be projecting to and innervating inappropriate alternate targets in the telencephalon, which could provide added insights into which pathfinding cues are used to direct dHb axons.

Given the recent effort to understand the brain structures and neuropathology that underlie psychological diseases, and the knowledge that the size and morphology of the habenulae are reportedly altered in patients with bipolar and major depressive disorder (Savitz et al., 2011; Ranft et al., 2010), a deep understanding of habenular development is of benefit. The zebrafish is well suited to address many of the remaining questions thanks to the current availability of transgenes that allow for the observation and manipulation of select cell populations as well as the detection of cell signaling events. Furthermore, the advent of CRISPR technology will allow the field to generate targeted mutant or transgenic alleles as needed.

However, there are currently few methods to specifically alter early dHb development and connectivity. The *wls*, *fgf8a*, and *smo* mutants, which have defective

progenitor populations discussed earlier, are not homozygous viable and their phenotypes are highly pleiotropic. Zebrafish larvae mutant for *cxcr4b* have altered dHb efferent outgrowth, and homozygotes are viable; but, the *cxcr4b* phenotype also includes defects in the lateral line, olfactory neurons and retinal neurons, among others (Li et al., 2005; Miyasaka et al., 2007; Palevitch et al., 2010; Aman & Piotrowski, 2008). This pleiotropy renders mutant alleles unsuitable for behavioral studies or analysis of neural activity. Therefore, to produce tools capable of manipulating dHb development and connectivity, we require a better understanding of how the dHb are formed. For example, if we can determine how and when specific neuronal populations arise from the *dbx1b*$^{+}$ progenitors or *cxcr4b*$^{+}$ precursors we should be able to more precisely alter their fate or function.

In summary, the work discussed in this thesis has provided new insights into how the habenular nuclei form. I have characterized a mutation in the zebrafish *wls* gene, and using this mutation, revealed a novel role for Wnt signaling in the generation of dHb progenitors. Study of the *wls* mutant helped to clarify the ambiguity surrounding the relative identities of *dbx1b*$^{+}$ progenitor and *cxcr4b*$^{+}$ precursor populations that contribute to the dHb. Although expression of *cxcr4b* was known to localize to cells that reside in the habenulae, no function had previously been attributed to the chemokine pathway in dHb development. My work has identified the Cxcr4b-chemokine pathway as a regulator of dHb axon outgrowth. Further, I have integrated the Wnt, Shh, Fgf, and chemokine signaling pathways into a larger network that underlies the formation of the dHb.

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SARA ROBERSON

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EDUCATION

2017 (expected) Ph.D., Johns Hopkins University, Baltimore, MD
Cell, Molecular, Developmental Biology, and Biophysics (CMDB) Department; thesis research performed at the Carnegie Institution for Science, Department of Embryology; thesis defended December, 2016

2010 B.Sc., Sewanee: The University of the South, Sewanee, TN
Major and Honors in Biology and Spanish

2008 Education Abroad, La Compultense de Madrid
Studied Spanish, medieval art, and architecture at La Compultense and on foot over 200 miles of the Camino de Santiago

RESEARCH

2012-2016 Graduate Honors Thesis
Signaling pathways underlying zebrafish habenular development and connectivity.
Determined the roles of and interactions between multiple morphogen pathways in generating the progenitor and precursor populations that give rise to the zebrafish habenulae, and investigating the role of chemokine signaling in habenular development.

2010 Biology Undergraduate Honors Thesis
Method for isolation and characterization of Eastern red spotted newt cardiolipin: differences in cardiolipin species in winter and summer acclimatized animals. One-year research project culminating in a thesis detailing a cardiolipin isolation method and novel information on the Eastern Red Spotted Newt and their ability to withstand winter temperatures by altering cardiolipin composition.

2010 Spanish Undergraduate Honors Thesis
Knights and Cavalry in Medieval Spain. Paper and presentation written and delivered in Spanish on the difference between myth and reality of knights and cavalry in medieval Spain.

2003-2006 Psychology Research Project
Reliability of the Therapeutic Alliance Scale for Adolescents with Borderline Personality Disorder in Dialectical Behavior Therapy. Completed over three

years culminating in a paper submitted to the INTEL Science Talent Search. Schneider Children's Hospital, New Hyde Park, NY

EXPERIENCE

2014-2015 Graduate Level Teaching Assistant, Johns Hopkins University, Baltimore, MD.

Used knowledge of developmental biology and experimental techniques to organize and hold office hours and review sessions, and to grade exams for the graduate level Genomes & Development course.

2012-2013 Teaching Assistant, Johns Hopkins University, Baltimore, MD.

Applied knowledge of genetics and developmental biology to teach an undergraduate genetics laboratory class and hold office hours to assist undergraduates with their genetics lecture course material

2010-2011 Horse Trainer and Farm Worker, Centaur Farms, Silver Creek, GA.

Used extensive knowledge of equestrian skills and horse management to provide care and training for stallions, mares, and foals at a warmblood dressage breeding and training farm

2010 Farm Manager, Barkwick Farms, Sewanee, TN.

Oversaw the care and feeding of all farm animals, coordinated veterinary visits, farrier visits and feed delivery to ensure smooth barn functioning and the health of the animals

2009-2010 Science Research Intern, The University of the South, TN, Sewanee, TN.

Performed research and learned scientific research techniques at Sewanee: The University of the South and Vanderbilt University that led to an honors thesis project, helping to understand why newts, unlike other cold-blooded animals, do not hibernate

2008-2009 Assistant to the Professor, The University of the South, Sewanee, TN.

Graded all student homework and laboratory assignments for an Introduction to Statistics course

2007 Summer Intern, Wildlife Conservation Society, Bronx, NY.

Applied Spanish language, computer, and writing skills to write a program-wide policy concerning digital photographs, as well as catalogued over 4,500 photographs and updated project fact sheets

HONORS AND AWARDS

2015 1st place Best Graduate Student Poster Presentation, Mid-Atlantic Regional SDB Meeting

2014 Best Student Poster, CMDB department retreat

2011 Thomas Hunt Morgan Award, Johns Hopkins University

2010 Summa cum laude, Sewanee: University of the South

2010 Inducted Phi Beta Kappa National Honor Fraternity

2007-2010 Order of Gownsmen (academic honors society and student governing body)

2006-2010 Dean's List

2006-2010 Chancellor's Scholarship (four-year academic merit full-tuition award)

2010 Schauss Award for Excellence in Spanish

2010 Earned Distinction on comprehensive exams (undergraduate exit exams covering four years-worth of a major's material) in Biology and Spanish (the only student to receive distinction in either of these majors for that year)

2008 Inducted into Sigma Delta Pi (Spanish honors society)

2006 Semifinalist for INTEL Science Talent Search

2006 Gold Award, Girl Scouts of America

PUBLICATIONS

Roberson, S., Halpern, M.E. *Invited review*. Signaling pathways underlying development and connectivity. Under preparation for *Seminars in Cell and Developmental Biology*.

Roberson, S., Halpern, M.E. *Under review*. Convergence of Signaling Pathways Underlying Habenular Formation and Axon Outgrowth.

Kuan, Y. S.*, **Roberson, S*.,** Akitake, C. M., Fortuno, L., Gamse, J., Moens, C., & Halpern, M. E. (2015). Distinct requirements for Wntless in habenular development. *Developmental Biology*, *406*(2), 117-128. *Co-first authors.

Rochard, L., Monica, S.D., Ling, I.T.C., Kong, Y., Roberson, S., Harland, R., Halpern, M.E., and Liao, E.C. (2016). Roles of Wnt pathway genes *wls, wnt9a, wnt5b, frzb* and *gpc4* in regulating convergent-extension during palate morphogenesis. *Development, 143*(14), 2541-2547.

PRESENTATIONS

2016 Roberson S. **Invited speaker**. Integration of multiple signaling pathways in habenular development and connectivity. Mid-Atlantic Regional Zebrafish meeting. National Institutes of Health, Bethesda MD.

2016 Roberson S., Halpern M.E. **Poster**. *Integration of multiple genetic pathways in habenular development.* The Allied Genetics Conference. Orlando, FL. July.

2016 Roberson S., Halpern M.E. **Poster**. Integration of multiple genetic pathways in habenular development. Mid-Atlantic Regional SDB meeting. Howard University, Washington DC.

2016 Roberson S., Halpern M.E. **Poster**. Integration of multiple genetic pathways in habenular development. Mid-Atlantic Regional Zebrafish Meeting, UPenn, Philadelphia, PA.

2015 Roberson S., Fuchsman A., Halpern M.E. **Poster**. Habenular precursors require Wntless-dependent Wnt signaling. 74th Annual Society for Developmental Biology Meeting, Snowbird, UT.

2015 Roberson S., Kuan Y.S., Fortuno L., Akitake C., Gamse J., Moens C., Halpern M.E. **Poster**. Multiple roles for Wntless in habenular development. Mid-Atlantic Regional Zebrafish meeting. Einstein University, Bronx, NY.

2015 Roberson S., Kuan Y.S., Fortuno L., Akitake C., Gamse J., Moens C., Halpern M.E. **Poster**. Multiple roles for Wntless in habenular development. Mid-Atlantic Regional SDB meeting. Princeton University, Princeton NJ.

2014 Roberson S., Kuan Y.S., Fortuno L., Akitake C., Gamse J., Moens C., Halpern M.E. **Poster**. Multiple roles for Wntless in habenular development. Mid-Atlantic Regional Zebrafish meeting. Temple University, Philadelphia, PA.

2014 Roberson S., Kuan Y.S., Fortuno L., Akitake C., Gamse J., Moens C., Halpern M.E. **Poster.** Discovering the role of Wntless in habenular development. International Conference on Zebrafish Development and Genetics. University of Wisconson, Madison, WI.

2014 Roberson S. **Invited speaker**. The role of Wntless in habenular development. Mid-Atlantic Regional SDB meeting. Johns Hopkins University, Baltimore, MD.

2013 Roberson S., Kuan, YS. Halpern M.E. **Poster**. The enigma of zebrafish Wntless. Mid-Atlantic Regional Zebrafish meeting. University of Maryland, Baltimore County, Baltimore, MD.

2013 Roberson S., Kuan, YS. Halpern M.E. **Poster**. Discovering the role of Wntless in habenular development. Mid-Atlantic Regional SDB meeting. The College of William & Mary, Williamsburg, VA.

MEMBERSHIPS

2011- present Society of Developmental Biology member

2016 Genetics Society of America

ACTIVITIES/COMMUNITY SERVICE

2012-present Environmental Conservation Reforestation Leader, Baltimore, MD.
Lead volunteers in tree planting events and educate volunteers about the value of reforestation in watershed ecosystems through the Gunpowder Valley. This work is done with the Gunpowder Valley Conservancy between six to eight times per year.

1993-present Lifetime member of the Girl Scouts of America

2012-2015 Graduate program recruitment weekend volunteer, Baltimore, MD.
Organize and run the zebrafish demonstration during the annual Cell, Molecular, Developmental Biology and Biophysics department recruitment event. Involves coordinating volunteers, managing embryonic fish stocks, and guiding prospective students through the zebrafish facility and microscope stations.

2015-present Member of the United States Dressage Federation (USDF).
National association and governing body of equestrians that participate in the sport of dressage.

2015-present Member of United States Equestrian Federation (USEF).
National association and governing body of all equestrian sports.

2014 Volunteer at Legacy Chase at Shawan Downs, Lutherville, MD.
Organized, checked-in, and dispatched volunteers at an equestrian event held in support of Greater Baltimore Medical Center (GBMC) Oncology.

2010 Pilgrim on the Camino de Santiago, Northern Spain.
Recipient of the "Compostela" for walking over 600 miles of the historically and culturally important Camino de Santiago pilgrimage.